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UTILITY PATENT APPLICATION TRANSMITTAL
(Small Entity)

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Docket No.
30406Total Pages in this Submission
3**TO THE ASSISTANT COMMISSIONER FOR PATENTS****Box Patent Application**
Washington, D.C. 20231

Transmitted herewith for filing under 35 U.S.C. 111(a) and 37 C.F.R. 1.53(b) is a new utility patent application for invention entitled:

CELL INTERNALIZED PEPTIDE-DRUG CONJUGATES

and invented by:

TERUNA J. SIAHAAN, HELENA YUSUF-MAKAGIANSAR, MEAGAN ANDERSON and RONG "CHRISTINE" XUIf a **CONTINUATION APPLICATION**, check appropriate box and supply the requisite information:☐ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application No.: _____

Which is a:

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Enclosed are:

Application Elements

1. ☒ Filing fee as calculated and transmitted as described below
2. ☒ Specification having 33 pages and including the following:
 - a. ☒ Descriptive Title of the Invention
 - b. ☐ Cross References to Related Applications (if applicable)
 - c. ☐ Statement Regarding Federally-sponsored Research/Development (if applicable)
 - d. ☐ Reference to Microfiche Appendix (if applicable)
 - e. ☒ Background of the Invention
 - f. ☒ Brief Summary of the Invention
 - g. ☒ Brief Description of the Drawings (if drawings filed)
 - h. ☒ Detailed Description
 - i. ☒ Claim(s) as Classified Below
 - j. ☒ Abstract of the Disclosure

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Application Elements (Continued)

3. ☒ Drawing(s) *(when necessary as prescribed by 35 USC 113)*
a. ☒ Formal b. ☐ Informal Number of Sheets 13 + 2 sets of photographs
4. ☒ Oath or Declaration
a. ☒ Newly executed *(original or copy)* ☐ Unexecuted
b. ☐ Copy from a prior application (37 CFR 1.63(d)) *(for continuation/divisional application only)*
c. ☒ With Power of Attorney ☐ Without Power of Attorney
d. ☐ DELETION OF INVENTOR(S)
Signed statement attached deleting inventor(s) named in the prior application,
see 37 C.F.R. 1.63(d)(2) and 1.33(b).
5. ☐ Incorporation By Reference *(usable if Box 4b is checked)*
The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.
6. ☐ Computer Program in Microfiche
7. ☒ Genetic Sequence Submission *(if applicable, all must be included)*
a. ☒ Paper Copy
b. ☒ Computer Readable Copy
c. ☒ Statement Verifying Identical Paper and Computer Readable Copy

Accompanying Application Parts

8. ☒ Assignment Papers *(cover sheet & documents)*
9. ☐ 37 CFR 3.73(b) Statement *(when there is an assignee)*
10. ☐ English Translation Document *(if applicable)*
11. ☐ Information Disclosure Statement/PTO-1449 ☐ Copies of IDS Citations
12. ☐ Preliminary Amendment
13. ☒ Acknowledgment postcard
14. ☒ Certificate of Mailing
☐ First Class ☒ Express Mail *(Specify Label No.):* EL618531779US

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Accompanying Application Parts (Continued)

15. ☐ Certified Copy of Priority Document(s) (if foreign priority is claimed)
16. ☒ Small Entity Statement(s) - Specify Number of Statements Submitted: one
17. ☐ Additional Enclosures (please identify below):

Assignment Recordation Sheet; Assignment; \$40.00; Petition to Accept Colored Photographs;
\$130.00


Fee Calculation and Transmittal

CLAIMS AS FILED

For	#Filed	#Allowed	#Extra	Rate	Fee
Total Claims	34	- 20 =	14	x \$9.00	\$126.00
Indep. Claims	5	- 3 =	2	x \$39.00	\$78.00
Multiple Dependent Claims (check if applicable) <input type="checkbox"/>					\$0.00
BASIC FEE					\$345.00
OTHER FEE (specify purpose)					\$0.00
TOTAL FILING FEE					\$549.00

- ☒ A check in the amount of **\$549.00** to cover the filing fee is enclosed.
- ☒ The Commissioner is hereby authorized to charge and credit Deposit Account No. **19-0522** as described below. A duplicate copy of this sheet is enclosed.
- ☐ Charge the amount of _____ as filing fee.
- ☒ Credit any overpayment.
- ☒ Charge any additional filing fees required under 37 C.F.R. 1.16 and 1.17.
- ☐ Charge the issue fee set in 37 C.F.R. 1.18 at the mailing of the Notice of Allowance, pursuant to 37 C.F.R. 1.311(b).

Dated: **July 31, 2000**


Signature
Tracey S. Truitt, Reg. No. 43,205

cc:

Applicant or Patentee: TERUNA J. SIAHAAN, HELENA YUSUF-MAKAGIANSAR, MEAGAN ANDERSON, RONG "CHRISTINE" XU	Attorney's Docket No.: 30406
Serial or Patent No.:	
Filed or issued:	
For: CELL INTERNALIZED PEPTIDE-DRUG CONJUGATES	

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) AND 1.27(d)) - NONPROFIT ORGANIZATION**

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION: University of Kansas, Inc.

ADDRESS OF ORGANIZATION: Strong Hall, Lawrence, Kansas 66044

TYPE OF ORGANIZATION:

- ☒ [X] University or other institution of Higher Education
- ☐ [] Tax Exempt Under Internal Revenue Service Code (26 USC 501(a) and 501(c)(3))
- ☐ [] Nonprofit Scientific or Educational Under the Statute of State of the United States of America;
Name of State _____, Citation of Statute _____
- ☐ [] Would qualify as Tax Exempt Under Internal Revenue Service Code (26 USC 501(a) and 501(c)(3) if
located in the United States of America
- ☐ [] Would qualify as Nonprofit Scientific or Educational under Statute of the United States of America if
located in the United States of America; Name of State _____, Citation of Statute _____

I hereby declare that the above-identified nonprofit organization qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled LEUKOCYTE INTERNALIZED PEPTIDE-DRUG CONJUGATES by Inventor(s) TERUNA J. SIAHAAN, HELENA YUSUF-MAKAGIANSAR, MEAGAN ANDERSON and RONG "CHRISTINE" XU described in the specification filed herewith.

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above-identified invention. If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below*, and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person had made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

***NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)**


FULL NAME _____

ADDRESS _____

☐ [] INDIVIDUAL ☐ [] SMALL BUSINESS CONCERN ☒ [X] NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing therefrom, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING: Jim Pottorff	
TITLE OF PERSON OTHER THAN OWNER	
ADDRESS OF PERSON SIGNING Strong Hall, Lawrence, Kansas 66044	
SIGNATURE 	DATE 20 July 00

CELL INTERNALIZED PEPTIDE-DRUG CONJUGATES

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention is concerned with treating diseases involve ICAM-1 and LFA-1 proteins such as leukemia, Chron's disease, inflammation, asthma, rheumatoid arthritis, and other leukocyte related diseases. More particularly, the present invention is concerned with improving efficacy and reducing toxicity of drugs normally used to treat leukemia, Chron's disease, inflammation, asthma, rheumatoid arthritis, and other leukocyte related diseases. Still more particularly, the present invention is concerned with the conjugation of drugs with peptides which bind to cell adhesion receptors on cell surfaces of leukocytes, endothelial cells, and epithelial cells and are internalized by these cells. Finally, and most particularly, the invention is concerned with the conjugation of drugs with intercellular adhesion molecule-1 (ICAM-1) peptides and lymphocyte function-associated antigen-1 (LFA-1) peptides for drug delivery to the cytoplasmic domain of cells expressing ICAM-1 and LFA-1.

Description of the Prior Art

Leukocyte-related diseases often result from aberrant immune responses including reactions of leukocytes on "self" antigens. Such reactions contribute to autoimmune diseases including rheumatoid arthritis, insulin-dependent diabetes mellitus, and multiple sclerosis. Similarly, organ transplantation rejection results from leukocyte attack, specifically from T-cells. Accordingly inhibition of T-cell actions and their subsequent destruction aids in combating such diseases.

One way to modulate leukocyte immune response utilizes inhibitors of the ICAM-1/LFA-1 receptor interaction. For example, monoclonal antibodies (mAbs) to ICAM-1 and LFA-1 have been utilized to generate tolerance in immune response disorders such as allograft rejection (Kato et al., 1996; Nakamura et al., 1996), rheumatoid arthritis (Davis et al., 1995), and autoimmune encephalomyelitis (Willenborg et al., 1996). Despite the encouraging clinical results in inducing tolerance, such mAbs may be potentially immunogenic and trigger an effectiveness-limiting immunity. In addition, the formulation of antibodies is challenging and costly. Another way to modulate immune response utilizes small peptide fragments derived from ICAM-1 and LFA-1 sequences which inhibit ICAM-1/LFA-1 interaction (Ross

et al., 1992; Fecondo et al., 1993; Benedict et al., 1994; Siahaan et al., 1996). These peptides may have a better physicochemical stability than antibodies and may not possess any immunogenic properties. It has also been shown that a cyclic peptide (cIBR) derived from the sequence of ICAM-1 inhibits ICAM-1/LFA-1 interactions (Siahaan et al., 1996).

Furthermore, despite the ability to inhibit ICAM-1/LFA-1 interactions and attendant leukocyte-related diseases through treatment with antibodies, such treatments are typically ineffective over the long term due to their transient nature. Additionally, once the mAbs invoke an immune response, their effectiveness is severely limited.

When toxic drugs are used to kill the leukocytes and combat leukocyte-related diseases, many adverse side effects are encountered. These side effects include the non-selective killing of cells in addition to targeted cells as well as the suppression of the proliferation of healthy cells. Therefore, new methods which selectively target drugs to cells involved in the disease process will be beneficial to patients. For example, selectively targeting cytotoxic drugs to leukocytes will reduce drug toxicity and increase drug efficacy.

SUMMARY OF THE INVENTION

The present invention overcomes the problems inherent in the prior art and provides peptides derived from ICAM-1 and LFA-1 sequences which bind to receptors on leukocytes. These peptides are conjugated with drugs which are effective in treating leukocyte-related diseases. Such peptides and their conjugates are subsequently internalized by the leukocyte wherein the drug portion of the conjugate exerts toxic effects. Thus, the present invention provides an effective means of drug delivery to the cytoplasmic domain of leukocytes with improved efficacy and reduced toxicity in comparison to conventional methods of treatment. Because these peptides bind to specific receptors on the surface of the leukocytes, drug delivery is "targeted" to cells having such receptors. This greatly reduces toxic effects on other cells which, due to the non-selectivity of the drugs utilized, generally accompanies treatment of leukocyte-related diseases.

Similarly, these drug-peptide conjugates can be used to treat diseases related to increased expression of ICAM-1 on endothelial and epithelial cells such as inflammation, asthma, allergies and Chron's disease.

Peptides useful in the present invention include peptides which bind to LFA-1 or ICAM receptors on leukocytes. Preferably, the peptides are derived from ICAM-1 and LFA-1 sequences and from about 4-30 amino acid residues in length. Still more

preferably, such sequences will include from about 8-15 amino acid residues. Most preferably, such sequences will include from about 10-12 amino acid residues. Such sequences are linear and cyclic peptides derived from I-domain of LFA-1, including LAB, cLAB.L, cLAB.C, and cLAB.R which inhibit homotypic aggregation of a certain type of leukocyte, namely T-cells. These sequences are listed herein as SEQ ID Nos. 1-4. Other sequences include sequences derived from an ICAM-1 sequence, IB, listed herein as SEQ ID No. 5. The sequences derived from ICAM sequences are cyclized and include cIBL, cIBC, and cIBR, listed herein as SEQ ID Nos. 6-8, respectively.

Sequences including or having a sequence which has at least about 50% sequence identity with any one of SEQ ID Nos. 1-8 and which exhibits similar internalization properties are within the scope of the present invention. Preferably, such sequences will have at least about 60% sequence identity with any one of SEQ ID Nos. 1-8 and still more preferably at least about 75% sequence identity. Alternatively, sequences including or having a sequence which has at least about 50% sequence homology with any one of SEQ ID Nos. 1-8 and which exhibits similar internalization properties are embraced in the present invention. More preferably, such sequences will have at least about 60% sequence homology with any one of SEQ ID Nos. 1-8 and still more preferably at least about 75% sequence homology. Additionally, sequences which differ from any one of SEQ ID Nos. 1-8 due to a mutation event but which still exhibit similar properties are also embraced in the present invention. Such mutation events include but are not limited to point mutations, deletions, insertions and rearrangements.

One major complementary signal for sustaining leukocyte activation is the interaction between ICAM-1 (ICAM-1, also known as CD54) and LFA-1 (LFA-1, made up of a dimer comprising CD11a/CD18). Furthermore, the combination of ICAM-1/LFA-1 and B7/CD28 complexes modulates the duration and amplitude of leukocyte activation. These complexes induce the movement of actin molecules in the cytoskeleton, thereby producing the accumulation of surface receptors such as LFA-1 and ICAM-1 at the interface between antigen presenting cells (APC) and T-cells (Wülfing and Davis, 1998). When LFA-1 interacts with ICAM-2 (predominantly expressed on resting endothelium), its interaction with LFA-1 is more important for non-activated circulating T-cells. Meanwhile ICAM-3 is expressed by monocytes and resting lymphocytes and plays a major role in the initiation of the immune response (de Fougères et al., 1992).

LFA-1/ICAM-1 interaction requires the activation of LFA-1 (Dustin and Springer, 1989) which can be triggered by CD2, CD3, phorbol esters (i.e., phorbol 12-

myristate-13 acetate or (PMA)) and MHC class II molecules by stimulating protein kinase C (PKC) mechanisms (Rothlein and Springer, 1986; van Kooyk, et al., 1993). PMA activates LFA-1 molecules by directly activating PKC while anti-CD2 and anti-CD3 antibodies activate PKC by stimulating inositol phospholipid metabolisms (Fidgor et al., 1990). Divalent cations contribute to LFA-1/ICAM-1 interactions by enhancing the functional activity of the adhesion molecules (van Kooyk et al., 1993), and the ion type requirement relates to a specific domain of LFA-1 and ICAM-1 (Stanley and Hogg, 1998).

SEQ ID No. 2 was synthesized as a 12-residue peptide containing 10 amino acid residues (Ile²³⁷ – Gly²⁴⁶) from the “insert” (I)-domain of LFA-1 which is known to contain residues for ICAM-1 binding (Benedict et al., 1994). Penicillamine (Pen) and cysteine (Cys) residues were then added to the N- and C-termini (Benedict et al., 1994) to form cyclic peptides via a disulfide bond between the Pen1 and Cys12 residues. The formation of this cyclic peptide restricts the peptide conformation to produce a conformational stability, thereby providing better selectivity for cell surface receptors than its linear counterpart (Siahaan et al., 1996).

The resulting peptide, cLAB.L, inhibits T-cell aggregation by inhibiting ICAM-1/LFA-1 interaction (Siahaan *et al.*, 1996). There are two potential inhibitory mechanisms performed by this cyclic peptide: (a) inhibition of T-cell adhesion by binding to ICAM-1 and/or (b) disruption of α - and β -subunit dimerization by binding to the β -subunit of LFA-1. Because the heterodimeric formation of α - and β -subunits of LFA-1 is necessary for ICAM binding, disruption of this heterodimeric formation results in an inhibition of the ICAM-1/LFA-1 interaction.

Investigations leading to the present invention have also determined and characterized the cLAB.L binding sites. It was discovered that cLAB.L binds to the D1-domain of ICAM-1 as well as to ICAM-3 on the surface of T-cells. Binding of the cLAB.L to the D1-domain occurs more efficiently than its binding to ICAM-3. The binding characteristics of cLAB.L were evaluated using the FITC-labeled cLAB.L (FITC-cLAB.L) on activated Molt-3 cells. The FITC-cLAB.L binding to the cell surface-receptor is inhibited by the unlabeled-cLAB.L suggesting that the FITC-cLAB.L bound to the same receptor as unlabeled-cLAB.L. Moreover, the binding of FITC-cLAB.L exhibited bimodal cell-distribution suggesting the occurrence of multiple and dynamic states of activated ICAM receptors. Binding of the peptide to ICAMs (e.g. ICAM-1, ICAM-2, ICAM-3) was enhanced by the presence of a Ca^{2+} and Mg^{2+} mixture, thereby suggesting the involvement of divalent cations during peptide-ICAM interactions. Additionally, it was discovered that this peptide was internalized by

ICAM receptors on T-cells. Proof of this internalization was supported by the marked differences in peptide binding between 4°C and 37°C as well as by the presence of the peptide in the cytoplasm when observed by confocal-microscopy. This internalization of the peptide, when coupled with drug conjugation, permits delivery of drugs to the cytoplasmic domain of cells having ICAM receptors. Thus, the invention provides effective treatment of leukocyte-related diseases, because leukocytes have ICAM receptors.

It is known that the cyclic peptide (cIBR) derived from the sequence of ICAM-1 inhibits ICAM-1/LFA-1 interactions (Siahaan et al., 1996). It has now been discovered that this peptide binds to and is internalized by LFA-1 surface receptors of T-cells. Furthermore, it has also been determined that the conformation of this peptide plays an important role in its selectivity for the receptor. Such receptor internalization is believed to be a complementary mechanism of the inhibition of cell-cell adhesion.

Due to the internalization of peptides which bind to receptors on leukocytes, one such peptide, SEQ ID No. 8, was selected for conjugation experiments. To perform the conjugation experiments SEQ ID No. 8 was conjugated with drugs in order to evaluate resultant toxicity to targeted and non-targeted cells. Conjugation was accomplished by dissolving a quantity of drug in water and adding in an amount of peptide prior to storing the resultant solution overnight. Such conjugates bound to leukocytes expressing LFA-1 thereby interfering with ICAM/LFA-1 interactions. Binding to the LFA-1 receptors resulted in internalization of the peptide-drug conjugate into the cytoplasmic domain of cells wherein the drug portion of the conjugate exerted potent toxic affects. Advantageously, these toxic affects were localized to cells expressing LFA-1 on their surfaces, thereby delivering these drugs to a specific type of cell. Because only leukocytes express LFA-1 on their surfaces, drugs can be specifically delivered to the surfaces of leukocytes and internalized before exerting their toxic affects and without significant toxicity to other cells. The present invention therefore finds great utility in the treatment of leukocyte-related diseases. Preferably, diseases treatable by such peptide-drug conjugates include Chron's disease, asthma, inflammations, lupus, rheumatoid arthritis, multiple sclerosis, ulcerative colitis, pemphigus vulgaris, pemphigoid, allergies, HIV infections, and epidermolysis.

Thus, by conjugating peptides which are internalized with drugs, internalization of the conjugate by leukocytes contributes to targeted drug delivery with a high degree of specificity and toxicity for targeted cells and decreased toxicity for non-targeted cells. In the case of peptides derived from LFA-1 sequences which are conjugated with drugs, such conjugates are internalized by leukocytes and epithelial and endothelial

cells which have ICAM receptors on their surface. In the case of peptides derived from ICAM sequences which are conjugated with drugs, such conjugates are internalized by leukocytes which have LFA-1 receptors on their surface. Preferably, the peptides are conjugated with drugs effective in destroying leukocytes. Still more preferably, the conjugated peptides are LFA-1 peptides which can modulate the functions of epithelial and endothelial cells via mechanisms in the cytoplasm such as the mechanisms used by the asthma drugs albuterol and propidium. Preferably, the drug is selected from the group consisting of drugs classified as antiinflammatory agents, antitumor agents (i.e., intercalating agents, tubulin assembly inhibitors, alkylating agents), oligonucleotides, cytokines, enzyme inhibitors (i.e., ACE inhibitors, HIV-protease inhibitors; viral protease inhibitors), and vasoregulator agents. Still more preferably, the drug is selected from the group consisting of methotrexate, lovastatin, taxol, ajmalicine, vinblastine, vincristine, cyclophosphamide, fluorouracil, idarubicin, ifosfamide, irinotecan, 6-mercaptopurine, mytomycins, mitoxantrone, paclitaxel, taxol, pentostatin, plicamycin, topotecan, fludarabine, etoposide, doxorubicin, doxotaxel, danorubicin, albuterol, propidium. Therefore, the resultant peptide-drug conjugates both interfere with ICAM-1/LFA-1 interactions and destroy leukocytes once the conjugates are internalized. Furthermore, the internalized conjugates may regulate functions of epithelial and endothelial cells such as those present in long epithelial cells and vascular endothelial cells. This ability to regulate functions is due to the presence of ICAM-1 on epithelial and endothelial cells which bind to and internalize LFA-1 peptides.

Notably, the present invention reduces the toxicity of anti-tumor and immunosuppressive, and other drugs by increasing the selectivity of the drugs to the target cells. This selectivity also results in increased potency at lower dosage levels. As an example, a drug currently used to treat leukemia, methotrexate (MTX), was conjugated to the N-terminal of the cIBR peptide to yield a MTX-cIBR conjugate. The conjugate's potency and selectivity was then compared to that of MTX alone. The conjugate was shown to be more potent against leukemic cells (which have LFA-1 receptors) than MTX alone. Additionally, the conjugate was not effective in killing Madin-Darby Canine Kidney (MDCK) cells, which do not have LFA-1 receptors. The potency and selectivity of the conjugates represent a distinct advance in the state of the art as current treatments using MTX have severe side effects including suppression of the proliferation of other healthy cells. Due to the conjugates' increased potency and selectivity, patients will be able to receive lower dosage levels of drugs with fewer adverse side effects.

BRIEF DESCRIPTION OF THE DRAWINGS

The filing of this patent contains at least one drawing executed in color. Copies of this patent with color drawing(s) will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.

5 Figure 1 is a graph illustrating blocking by cLAB.L peptide on the binding of FITC-conjugate antibodies to ICAM-1, ICAM-3 and LFA-1;

10 Fig. 2 is a graph illustrating the distribution of Molt-3 cell-population in response to the binding to FITC-cLAB.L whereby cells were preactivated with PMA for 1 hour (a), and cells were preactivated with PMA for 48 hours (b), while distribution of Molt-3 cell population in response to the binding of FITC-conjugate antibody to domain D1 of ICAM-1 is shown in (c);

15 Fig. 3 is a graph illustrating the binding specificity of FITC-cLAB.L on population-1 and -2 of Molt-3 cells, indicated by the saturation profile of the FITC-cLAB.L binding (a), and the ability of unlabeled cLAB.L to block the binding of FITC-cLAB.L in concentration-dependent manner (b);

20 Fig. 4a is a graph illustrating proportions of cell population-1 and -2 in response to the duration of PMA activation;

25 Fig. 4b is a graph illustrating the effect of cell activation on the binding of 25 μ M FITC-cLAB.L to cell population-1;

30 Fig. 5a is a graph illustrating time-temperature dependence-profiles of 25 μ M FITC-cLAB.L binding on population-1;

 Fig. 5b is a graph illustrating the population-2 of Molt-3 cells;

 Fig. 5c is a graph illustrating the effect of cation addition on the intensity of FITC-cLAB.L binding to PMA-activated Molt-3 cells;

35 Fig. 6a is a confocal microscopy photograph illustrating microscopy of FITC-cLAB.L binding and internalization on a Molt-3 cell clump at 37°C;

 Fig. 6b is a confocal microscopy photograph of FITC-cLAB.L binding and internalization on a single Molt-3 cell at 37°C;

40 Fig. 6c is a confocal microscopy photograph of FITC-cLAB.L binding and internalization on a single Molt-3 cell at 4°C;

 Fig. 6d is a confocal microscopy photograph of surface projection of Molt-3 cell following 37°C incubation;

 Fig. 6e is a confocal microscopy photograph of the same area as in Fig. 6d and showing the distribution of the FITC-cLAB.L in the cytoplasm;

45 Fig. 6f illustrates a single confocal section showing a minimal distribution of the FITC-cLAB.L on the cell peripheral;

Fig. 7 is a schematic of two different forms of an MTX-cIBR conjugate in accordance with the present invention;

Fig. 8 is a schematic illustration of the internalization process of an MTX-cIBR conjugate in accordance with the present invention;

Fig. 9 is a graph of an MTT assay comparing the toxicities of different concentration of MTX-cIBR conjugates and MTX alone in accordance with the present invention; and

Fig. 10 is a comparative graph illustrating metabolic activity after treatment with MTX, cIBR, and MTX-cIBR conjugates.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

As used herein, the following definitions will apply: "Sequence Identity" as it is known in the art refers to a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, namely a reference sequence and a given sequence to be compared with the reference sequence. Sequence identity is determined by comparing the given sequence to the reference sequence after the sequences have been optimally aligned to produce the highest degree of sequence similarity, as determined by the match between strings of such sequences. Upon such alignment, sequence identity is ascertained on a position-by-position basis, e.g., the sequences are "identical" at a particular position if at that position, the nucleotides or amino acid residues are identical. The total number of such position identities is then divided by the total number of nucleotides or residues in the reference sequence to give % sequence identity. Sequence identity can be readily calculated by known methods, including but not limited to, those described in: Computational Molecular Biology, Lesk, A. N., ed., Oxford University Press, New York (1988); Biocomputing: Informatics and Genome Projects, Smith, D.W., ed., Academic Press, New York (1993); Computer Analysis of Sequence Data, Part I, Griffin, A.M., and Griffin, H. G., eds., Humana Press, New Jersey (1994); Sequence Analysis in Molecular Biology, von Heinge, G., Academic Press (1987); Sequence Analysis Primer, Gribskov, M. et al., eds., M. Stockton Press, New York (1991); and Carillo, H., et al. Applied Math., 48:1073 (1988), the teachings of which are incorporated herein by reference. Preferred methods to determine the sequence identity are designed to give the largest match between the sequences tested. Methods to determine sequence identity are codified in publicly available computer programs which determine sequence identity between given sequences. Examples of such programs include, but are not limited to, the GCG program package (Devereux, J., et al., Nucleic Acids Research, 12(1):387 (1984)),

BLASTP, BLASTN and FASTA (Altschul, S. F. et al., J. Molec. Biol., 215:403-410 (1990). The BLASTX program is publicly available from NCBI and other sources (BLAST Manual, Altschul, S. et al., NCVI NLM NIH Bethesda, MD 20894, Altschul, S. F. et al., J. Molec. Biol., 215:403-410 (1990), the teachings of which are incorporated herein by reference). These programs optimally align sequences using default gap weights in order to produce the highest level of sequence identity between the given and reference sequences. As an illustration, by a polynucleotide having a nucleotide sequence having at least, for example, 95% "sequence identity" to a reference nucleotide sequence, it is intended that the nucleotide sequence of the given polynucleotide is identical to the reference sequence except that the given polynucleotide sequence may include up to 5 point mutations per each 100 nucleotides of the reference nucleotide sequence. In other words, in a polynucleotide having a nucleotide sequence having at least 95% identity relative to the reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence. Analogously, by a polypeptide having a given amino acid sequence having at least, for example, 95% sequence identity to a reference amino acid sequence, it is intended that the given amino acid sequence of the polypeptide is identical to the reference sequence except that the given polypeptide sequence may include up to 5 amino acid alterations per each 100 amino acids of the reference amino acid sequence. In other words, to obtain a given polypeptide sequence having at least 95% sequence identity with a reference amino acid sequence, up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 5% of the total number of amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino or the carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in the one or more contiguous groups within the reference sequence. Preferably, residue positions which are not identical differ by conservative amino acid substitutions. However, conservative substitutions are not included as a match when determining sequence identity.

Similarly, "sequence homology", as used herein, also refers to a method of determining the relatedness of two sequences. To determine sequence homology, two or more sequences are optimally aligned as described above, and gaps are introduced if necessary. However, in contrast to "sequence identity", conservative amino acid substitutions are counted as a match when determining sequence homology. In other words, to obtain a polypeptide or polynucleotide having 95% sequence homology with a reference sequence, 95% of the amino acid residues or nucleotides in the reference sequence must match or comprise a conservative substitution with another amino acid or nucleotide, or a number of amino acids or nucleotides up to 5% of the total amino acid residues or nucleotides, not including conservative substitutions, in the reference sequence may be inserted into the reference sequence.

A "conservative substitution" refers to the substitution of an amino acid residue or nucleotide with another amino acid residue or nucleotide having similar characteristics or properties including size, charge, hydrophobicity, etc., such that the overall functionality does not change significantly.

"Isolated" means altered "by the hand of man" from its natural state., i.e., if it occurs in nature, it has been changed or removed from its original environment, or both. For example, a polynucleotide or polypeptide naturally present in a living organism is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein.

"Drug" means any natural or artificially made chemical for use in the diagnosis, cure, mitigation, treatment, or prevention of illness or disease.

"Targeted cell" means a cell of a specific type or class which exhibits certain physical or functional characteristics and which is of interest due to these characteristics. In the case of medical treatment, when certain types of cells are involved in a disease-type process, treatment can be improved if such cells can be selectively treated without significantly affecting other cells which are not involved in the disease process. Thus, offending cells would be "targeted" and subject to selective treatment while other cells were unaffected.

Similarly, a "non-targeted cell" means a cell which does not have a physical or functional characteristic of a "targeted cell." Preferably, non-targeted cells are not significantly affected during medical treatment.

EXAMPLES

The following examples set forth preferred embodiments of the present invention. It is to be understood, however, that these examples are provided by way

of illustration and nothing therein should be taken as a limitation upon the overall scope of the invention.

EXAMPLE 1

This example confirmed that a peptide derived from LFA-1 inhibited ICAM-1/LFA-1 interaction by binding to ICAM sequences. Furthermore, it was determined that this peptide was internalized by leukocytes after binding to ICAM. Thus, peptides such as cLAB.L may be used as a convenient shuttle source to the interior of the leukocyte, thereby providing new methods of treating leukocyte-related diseases. In addition, these peptides can also be used to shuttle drugs to the other cells expressing ICAM-1 such as endothelial and epithelial cells.

Materials and Methods:

Cell Culture and Cell activation. Molt-3 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in suspension in RPMI1640 supplemented with 10% heat-inactivated fetal bovine serum and 100 mg/L of penicillin/streptomycin. Cells were grown or activated in 75-cm² tissue culture flasks (Corning) at 37°C in a saturating humidified atmosphere of 95% air and 5% CO₂. For the purpose of cell activation, phorbol 12-myristate-13-acetate (PMA) from Sigma (St. Louis, MO) or anti-CD3 antibody from Chemicon (Temecula, CA) was used in concentrations of 0.2 μM and 10 μg/mL, respectively. Incubation with PMA was varied (1, 2, 4, 16, 24, 40 and 48 hours) to study the affect of time on receptor activation. In other cases, periods of PMA activation for 4 hours and 48 hours were compared in their effects on cLAB.L binding to the cells.

Peptide Labeling. 50 mg of cLAB.L peptide was dissolved in a minimal amount of Milli-Q water and added with FITC at two times the molarity of the peptide. The FITC is reacted to the N-terminus of cLAB.L. The pH mixture was adjusted to 9.0 with 1 N NaOH and the reaction was run for 1 hour. The pH was then brought to 7.0 by adding 10% acetic acid. The mixture was lyophilized and purified using preparative reversed-phase (RP) HPLC. The molecular weight of the fraction containing FITC-cLAB.L was confirmed by fast atom bombardment (FAB) mass spectrometry to give M+1=1586.

Peptide Binding Experiment. Activated or non-activated cells were centrifuged at 2000 rpm for 5 minutes and resuspended in serum-free medium to reach a concentration of 3.5×10^6 /mL. Peptide stock solution was prepared in phosphate buffer saline (PBS). Serum-free medium of RPMI1640 was used for the binding experiment in a 48-well cell culture cluster (Corning) into which the mixture of cell

suspension, medium, and peptide solution was 400 μ L/well. The mixture was incubated at either 4°C or 37°C. In time-temperature dependence experiments, a sample was taken every 30 minutes for up to 4 hours incubation time. In a concentration-dependence experiment, incubation was carried out for 2 hours. In experiments to block FITC-cLAB.L binding, cLAB.L was added to the cell suspension followed by incubation for 1 hour at 4°C. The FITC-cLAB.L was then added followed by another hour of incubation. Cell suspensions without peptide addition were used as controls throughout the experiments.

At the end of incubation, cell suspensions were centrifuged at 3000 g for 3 minutes before being decanted and rinsed with 10 mM Hepes/PBS. The cell pellet was fixed using 4% paraformaldehyde/PBS for 20 minutes at room temperature. The mixture was then washed twice with 10 mM Hepes/PBS and resuspended in PBS. Samples were analyzed with a flow cytometer (Becton Dickinson). As many as 10,000 cells were counted for every sample, and each experiment was done at least in triplicate. Peptide binding affinity was represented by the average of median values of fluorescence intensity (FI), or in some cases, by its ratio to the relevant standard condition/reference.

Antibody Binding Experiment. A one step-direct method using FITC-labeled anti-LFA-1, anti-ICAM-1 or anti-ICAM-3 was applied to examine the ability of cLAB.L to block antibody binding to Molt-3 cells. The mAbs to LFA-1 tested were anti-human CD11a clone 38 and anti-human CD18 clone IB4 from Ancell (Bayport, MN). Another pair of clones (DF1524 and YFC118.3 from Accurate (Westbury, NY)) of anti-LFA-1 mAb that have been proven to inhibit aggregation and adhesion were also tested. Clone 15.2 and clone 8.4A6 of anti-ICAM-1 mAbs (Ancell), that recognize D1- and D2-domain of ICAM-1, respectively, were also used in this study. For ICAM-3, clone 186-2G9 which recognized the D1-domain was used to identify the peptide-ICAM-3 recognition. Peptide solution was incubated with Molt-3 cells for 1 hour prior to the addition of FITC-antibody. The flow cytometer analysis was carried out as described for the peptide binding experiment. A cyclic Arg-Gly-Asp (RGD) peptide (cyclo-(2,10)-Ac-Gly-Pen-Gly-His-Arg-Gly-Asp-Leu-Arg-Cys-Ala-NH₂) (SEQ ID No. 9) was used as a control peptide throughout the antibody binding experiments because LFA-1 does not recognize the tripeptide sequence Arg-Gly-Asp (SEQ ID No. 10) unlike other integrins (i.e., β_3 integrins: $\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$ and β_1 integrins). Furthermore, ICAM-1 does not contain an RGD sequence (Detmer and Wright, 1988).

Fluorescence and Confocal Microscopy Studies. Samples from peptide-binding experiments at 4°C and 37°C were photographed using fluorescence

microscope Nikon Eclipse TE300 and confocal microscope (Biorad MRC100 Laser Scanning Confocal Imaging System connected to Nikon Diaphot 2000 microscope).

Inhibition of Antibody Binding to LFA-1, ICAM-1 and ICAM-3 by cLAB.L. To determine whether the peptide binds to LFA-1, ICAM-1 or ICAM-3, the ability of the cLAB.L peptide to inhibit binding of FITC-labeled mAbs to LFA-1, ICAM-1 and ICAM-3 was evaluated and a cyclic RGD peptide was used as a negative control (Fig. 1). Two peptide concentrations, 80 and 160 μ M, were used in this inhibition study. Two anti-ICAM-1 mAbs directed toward the D1- and D2-domains and an anti-ICAM-3 antibody directed toward the D1-domain of ICAM-3 were used to evaluate the binding properties of cLAB.L. Two pairs of mAbs to LFA-1 were also tested in this experiment. One pair of mAbs was anti-CD11a clone 38 and anti-CD-18 clone IB4, neither of which inhibit cell adhesion. The other pair was anti-CD11a clone DF1524 and anti-CD18 clone YFC118.3, which both inhibit cell adhesion.

Fig. 1 shows the results for these studies. For a negative control, cyclic RGD peptide at 80 and 160 μ M had no effect on the binding of all the tested mAbs (see controls as the representatives). In the figure, LFA-1sp indicates the use of antibody specifically described by the manufacturer as an inhibitor of cell adhesion/aggregation. Fluorescence data were normalized between fluorescence of FITC-antibodies in the presence of cyclic-RGD peptide (control peptide) and the fluorescence of cells only. Both control peptide and cLAB.L were tested at two different concentrations shown on Fig. 1 as * and ** for 80 and 160 μ M respectively. The data shown represent the mean \pm S.E. of six determinations. The use of blank bars is to show that no blocking was observed. Binding of anti-ICAM-1 mAbs to D1-domain was blocked by cLAB.L peptide in a concentration dependent manner. However, the cLAB.L peptide did not inhibit the binding of anti-ICAM-1 antibody to the D2-domain. This result indicates that the cLAB.L peptide binds to the D1-domain but not D2-domain of ICAM-1. Interestingly, the cLAB.L peptide can also bind to the D1-domain of ICAM-3 because it can block the binding of anti-ICAM-3 binding to D1-domain. The cLAB.L peptide at a concentration of 160 μ M blocks the binding of anti-ICAM-1 (D1) and anti-ICAM-3 (D1) 59% and 74%, respectively. Thus, cLAB.L prefers binding to ICAM-1 over ICAM-3 thereby indicating that the peptide's major mechanism of activity is inhibition of the ICAM-1/LFA-1 interaction. The cLAB.L peptide can weakly inhibit the binding of anti-CD11a DF1524, presumably due to the recognition of cLAB.L by this anti-CD11a antibody. Furthermore, the cLAB.L peptide prevents the binding of antibody to CD11a on T-cells. In other words, the same sequence as cLAB.L in CD-11a is recognized by this anti-CD11a DF1524 antibody. The cLAB.L peptide did not block

the binding of other anti-CD11a and anti-CD-18 antibodies. This result indicates that cLAB.L may not bind to CD-18 to disrupt the CD11a-CD18 dimerization.

Binding Specificity of FITC-cLAB.L. As illustrated in Figs. 2a and 2b, the flow cytometry experiments show that FITC-cLAB.L binds to two populations (population-1 and -2) of Molt-3 cells. For these experiments, the peptide concentration was 25 μ M and the antibody concentration was used at the dilution suggested by the manufacturer. In contrast, FITC-labeled mAbs to ICAM-1 or LFA-1 show binding to only one cell population. This is illustrated in Fig. 2c. For FITC-cLAB.L peptide, the first population (population-1) has a high number of cells with low fluorescence intensity while the second population (population-2) has low number of cells but high fluorescence intensity (Figs. 2a and b). This indicates that the receptors in population-2 have a higher affinity than those in population-1.

In order to characterize receptor-mediated binding specificity of FITC-cLAB.L, two different experiments were performed. The first experiment tested concentration-dependent blocking of FITC-cLAB.L binding by the unlabeled cLAB.L. The second experiment tested FITC-cLAB.L binding saturation to the receptors on Molt-3 cells. Results of these experiments are given in Figs. 3a and 3b. The FITC-cLAB.L binding on 4 hour-preactivated cells approached saturation at around 400 μ M and 800 μ M on cell populations -1 and -2, respectively. These results are given in Fig. 3a. To examine the blocking ability of unlabeled cLAB.L, two different preactivated cells (i.e., 4 hour and 48 hour activation) were used. These results are shown in Fig. 3b. In Fig. 3a, a comparison was made between cells preactivated with PMA for 4 hours and 48 hours. The percentage of blocking by unlabeled peptide in Fig. 3b was taken as the relative fluorescence values to the binding of 25 μ M FITC-cLAB.L without the addition of unlabeled peptide (ratio of unlabeled and labeled peptide = 0). Each value in Fig. 3a represents the mean of fluorescence values relative to the reference (binding of 3.12 μ M FITC-cLAB.L) \pm S.E. Four determinations were carried out for both experiments which are illustrated in Figs. 3a and 3b. Error bars are missing when less than the size of a symbol. A blocking experiment was carried out by incubating the cells with unlabeled cLAB.L prior to the addition of FITC-cLAB.L. This reaction was run at 4°C to minimize peptide internalization (see below). The results show that the unlabeled cLAB.L inhibits the binding of FITC-cLAB.L on cell populations -1 and -2 in a concentration-dependent fashion (Fig. 3b). For population-1, both cell activation times at 4 hours and 48 hours produce comparable inhibitory results. On the other hand, the population-2 of 48 hour-preactivated cells were more prone to blocking by unlabeled

peptide than those of 4 hour preactivation. These results indicate that the 4 hour activation provides a higher affinity state of the receptors.

Effect of Activation Time and Activators on FITC-cLAB.L Binding. The number of cells and fluorescence intensities between the two populations were changed at various time of PMA activation. The fluorescence intensity in population-1 increased sharply in the first four hours of PMA activation, and then reached a steady state at 40 hours time of activation. At the same time, the cell number of population-2 increased with increasing activation time while the cell number in population-1 decreased proportionately (Fig. 4a). Each value shown in Fig. 4a represents the mean \pm S.E. of four determinations. The fluorescence intensity from the binding of FITC-cLAB.L (50 μ M) to PMA-activated cells was three- to six-fold higher than binding to the non-activated cells. Such a difference indicates that cell activation is crucial for peptide binding.

The effect of temperature and different activators (i.e., PMA, anti-CD3 and both Ca^{2+} and Mg^{2+}) on FITC-cLAB.L binding to the receptors on the activated Molt-3 cells was evaluated. The results for population-1 are shown in Fig. 4b and similar observations were found for population-2. Binding of the FITC-cLAB.L to non-activated Molt-3 cells at 4°C and 37°C was used as a negative control. The binding experiment was carried out in the presence of activating molecules anti-CD3, PMA, or divalent cations ($\text{Ca}^{2+} + \text{Mg}^{2+}$ at final concentration of 1.5 mM) at 4°C or 37°C. Peptide binding on non-activated cells at 4°C was used as reference. The data shown represent the mean of fluorescence values relative to the reference \pm S.E. of four determinations. Error bars are missing when less than the size of symbol. As shown in Figs. 5a and 5b, peptide binding at 4°C and 37°C was compared using cells preactivated with PMA for 4 hours or 48 hours. Again, each value represents the mean of fluorescence value \pm S.E. of four determinations and error bars are missing when less than the size of symbol. The binding reactions were monitored over a four hour period. PMA and CD3 activated Molt-3 cells bind to the peptide with higher affinity than the non-activated cells at 4°C and 37°C. The fluorescence intensities increased over the four hour time period when the experiments were performed at 37°C. However, the binding of the peptide was suppressed at 4°C thereby indicating that at 37°C, the peptide was internalized by the receptors. In contrast, addition Ca^{2+} and Mg^{2+} mixture to the non-activated cells was unable to promote peptide binding. These results indicate that the divalent cations only affected binding of the peptide to the activated cells as shown in Fig. 5c. For this experiment, the final concentrations of Ca^{2+} , Mg^{2+} , and $\text{Ca}^{2+} + \text{Mg}^{2+}$ were 1.5 mM. The control was compared to the intensity of FITC-cLAB.L binding to

PMA-activated cells without added cations. The data shown represent the mean of fluorescence values relative to the control \pm S.E. of three determinations. Error bars are not shown due to very small values.

Effects of Temperature and Divalent Cations on FITC-cLAB.L Binding

Affinity. The effect of activation times and temperature on the FITC-cLAB.L binding to the Molt-3 cells was also evaluated in order to study the relationship of the activated state of the receptors (i.e., ICAM-1) and activation time. The cells were preactivated with PMA for 4 hours and 48 hours. Binding of FITC-cLAB.L to the cells was sampled every 30 minutes for 240 minutes at 4°C and 37°C. Fluorescence intensities of binding for 30 minutes and 240 minutes incubation time are shown in Figs. 5a and 5b. There are two consistent patterns shown by both population-1 and -2. First, the fluorescence intensity of peptide binding was lower at 4°C than at the 37°C incubation. This result was also confirmed by fluorescence microscopy (Figs. 6a-c). For each of the photos in Fig. 6a-c, the fluorescence and confocal-fluorescence microscopes were used to observe surface binding and internalization respectively. Fig. 6a shows the fluorescence microscopy of Molt-3 cell-clump after incubation at 37°C. Cell population-1 was more susceptible to suppression by low temperature than is cell population-2. Furthermore, flow cytometry of samples taken every 30 minutes showed a more noticeable onset of saturation of binding in population-1 than population-2. These results provide support for the idea that the peptide is internalized by a receptor-mediated process, and population-1 is more sensitive to the energy-dependent internalization than population-2. Additionally, the peptide has a better affinity to both populations at 4 hour activation compared to the 48 hour activation, indicating that the activated state of the receptors diminished with time.

ICAM-1/LFA-1-mediated cell adhesion is dependent on divalent cations, temperature and an intact microfilamentous cytoskeleton (Detmer and Wright, 1988). Therefore, the effect of divalent cations on binding of FITC-cLAB.L to the PMA-activated cells was evaluated. These results are shown in Fig. 5c. A mixture of Mg^{2+} and Ca^{2+} improved binding of FITC-cLAB.L 3.4-fold and 1.8-fold to population-1 and -2, respectively, thereby demonstrating that divalent cations have higher influence on the FITC-cLAB.L binding to the receptors in cell population-1 than the receptors in population-2. Similarly, addition of Ca^{2+} cations alone caused a 1.5-fold binding enhancement of FITC-cLAB.L on population-1, while no effect was detected on population-2. No peptide binding was observed with the addition of Mg^{2+} alone. However, Mg^{2+} addition did increase the cell numbers of population-2 (data not

shown). These results demonstrate that binding of cLAB.L to the surface receptor is influenced by the synergistic effects of Ca^{2+} and Mg^{2+} cations.

Microscopy of Peptide Binding and Internalization. In addition to flow cytometer analysis, binding and internalization of FITC-cLAB.L at 4°C and 37°C was examined using fluorescence microscope (Figs. 6a-c) and confocal-fluorescence microscope (Figs. 6d-f). Surface binding of FITC-cLAB.L on an aggregate of Molt-3 cells is indicated by the green fluorescence with fluorescence microscope (Fig. 6a), and by the white to grey color with confocal-fluorescence microscope (Fig. 6d). Observation on single cells clearly showed a higher fluorescence on the cell from the 37°C binding experiment (Fig. 6b) than that of the 4°C binding experiment (Fig. 6c). Sectional images of cells from the two different temperatures indicate that peptide internalization took place at 37°C (Fig. 6e), but was almost negligible at 4°C (Fig. 6f).

Discussion

These experiments evaluated the mechanisms of action of cLAB.L peptide [cyclo-(1,12)-Pen-Ile-Thr-Asp-Gly-Glu-Ala-Thr-Asp-Ser-Gly-Cys-OH] (SEQ ID No. 2) in inhibiting ICAM-1/LFA-1-mediated homotypic T-cell adhesion. As mentioned above, there are two possible mechanisms of action for this peptide in inhibiting homotypic T-cell adhesion. One possibility is that the cLAB.L binds to ICAMs (ICAM-1 and ICAM-3) to inhibit ICAMs/LFA-1 interaction. The second possibility is that the cLAB.L peptide can also bind to the β -subunit (CD18) of LFA-1, thereby disrupting the integrity of the LFA-1 and inhibiting the ICAMs/LFA-1 interactions.

From these experiments, it was determined that cLAB.L peptide primarily binds to the D1 domain of ICAM-1. To a lesser extent, the cLAB.L peptide also binds to the D1 domain of ICAM-3 (Fig. 1). These results are supported by the ability of this peptide to inhibit anti-ICAM-1 and anti-ICAM-3 antibodies binding to the D1 domain but not D2 domain. A weaker blocking effect of cLAB.L for binding of anti-ICAM-3 compared to anti-ICAM-1 may be due to the higher sequence and/or conformational selectivity of cLAB.L for ICAM-1 than ICAM-3. Although 52% homology between ICAM-1 and ICAM-3 is largely contributed by domain D2 (77%), many studies have pointed out that the important residues for LFA-1 binding to ICAM-3 is in domain D1 (Holness, 1995; Bell et al., 1998). Thus, it is the non-conserved residues in D1-domain of ICAM-3 which contribute to integrin binding. These results also emphasize that the ICAM-1/LFA-1 interaction serves a different function than the ICAM-3/LFA-1 interaction.

Results with cLAB.L were also consistent with the previous study of a 9-amino acid linear peptide (TDGEATDSG), provided herein as SEQ ID No. 11. This peptide has sequence similarity with cLAB.L and inhibited binding of Mg^{2+} /EGTA-activated, but not phorbol 12, 13-dibutyrate-activated T-cells, to ICAM-1 soluble protein. This peptide was also the most potent peptide found from the I-domain (McDowall et al., 1998). This linear peptide was shown to reduce binding of T-cells to ICAM-1 to 70% with 2 mM of linear peptides which is higher than the concentrations (80 and 160 μ M) of cLAB.L used to inhibit the binding of ICAM-1-antibody in this work. This indicates that the conformational rigidity of the cyclic peptide cLAB.L contributes to its binding selectivity (Benedict et al., 1994) to ICAM-1.

The cLAB.L peptide was also found to inhibit binding of anti-LFA-1 antibody to the I-domain (Fig. 1). This was due to the recognition of cLAB.L by the anti-LFA-1 antibody because this peptide was derived from the I-domain of the LFA-1. In contrast, this peptide cannot inhibit antibody binding to the β -subunit and other α -subunit of LFA-1 (Fig. 1). These results suggest that the primary mechanism of binding of this peptide is via the ICAM-1 D1 domain while weakly binding via the ICAM-3 D1 domain but not to LFA-1.

The characteristics of blocking of unlabeled cLAB.L (Fig. 3a), saturation profile (Fig. 3b), and marked differences in binding between 4°C and 37°C (Fig. 5a and 5b) of FITC-cLAB.L support the validity of FITC-cLAB.L as a model to study the binding of cLAB.L. The binding of FITC-cLAB.L featured a two-population distribution and responded to the duration of cell activation (Figs. 2 and 4a), thus suggesting the occurrence of multiple and dynamic states of ICAM-1 and/or ICAM-3. A similar bimodal expression was observed as a result of LFA-1 activation on T-cells with a population ratio 2.5-to-1 (Kurzinger et al., 1981; Sanders et al., 1988). In another case of $\alpha_5\beta_1$ integrin, integrin activation produces two or more different conformational states reflected in distinct stages in adhesion signaling (Faull et al., 1993; García et al., 1998). A similar explanation may be used for FITC-cLAB.L binding to ICAMs. The short (4 hour) and long (48 hour) activation times produced two different activated states of ICAMs with different affinities for FITC-cLAB.L. Longer PMA activation time (48 hour) gave a higher number of cell population-2 than a 4 hour PMA activation time. The activated state of ICAMs in population-2 has higher affinity than that shown in population-1. Population-2 may have an activated ICAM state which forms clusters with high affinities for FITC-cLAB.L peptide. On the other hand, population-1 has activated ICAMs which spread out through the cell surface with lower affinities for

FITC-cLAB.L than for that of in population-2. Thus, a longer activation time gave a change for membrane receptor and cytoskeleton rearrangements.

There are at least two possible explanations for why PMA activation modulates the binding of LFA-1 peptide (cLAB.L) in this case despite the suggested constitutive-avidity of ICAM-1 to LFA-1. The first possible explanation is an indirect mechanism of activation based on the facts that cell-cell adhesion via adhesion molecules consists of a multistep process involving a cascade of recognition and conformational states. In relation to this event, it is apparent that PMA activation of Molt-3 cells triggers other cellular mechanisms such as redistribution of some components of plasma membrane or release of receptors from cytoskeleton which subsequently potentiate the binding of cLAB.L to ICAM-1. Reference may be made to the observed redistribution of lipid packing of the plasma membrane (Smith et al., 1993; Del Buono et al., 1996) and the postulation on the release of receptors from cytoskeletal constraint (Kucik et al., 1996) due to cell activation. The second possible explanation is that an active LFA-1, resulting from PMA stimulation, directly triggers a more avid ICAM-1 in Molt-3 cells. This explanation may be justified by the generality that T-cell stimulation could lead to induction of ICAM-1 on antigen-presenting cells. The use of PMA has an effect in increasing the binding of JY and SKW3 cells to purified LFA-1 within 0-200 sites μm^{-2} of LFA-1 (Dustin and Springer, 1989), thus indicating a response of ICAM-1 on the surface of T-cells to PMA stimulation. Others have also used PMA to induce ICAM-1 expression in epithelial cells (Bloemen et al., 1993) and shown that PMA stimulation may cause a rapid and transient phosphorylation on serine residue of ICAM-3 (Lozano et al., 1992).

Temperature and other activators can influence binding properties of FITC-cLAB.L peptide to T-cells. For example, anti-CD3 antibody produced the same effect as PMA. However, divalent cations alone did not activate binding between FITC-cLAB.L and ICAMs. For PMA and anti-CD3 antibody, the fluorescence intensity of FITC-cLAB.L is higher at 37°C than at 4°C in both populations. Thus, FITC-cLAB.L was internalized by ICAM receptors because low temperature (4°C) suppressed the ATP-dependent receptor-internalization. Using fluorescence microscopy, the binding of the labeled peptide is higher at 37°C than at 4°C (Figs. 6a-cs). Furthermore, internalization of this peptide was confirmed by the sectional images from confocal microscopy (Figs. 6d-f). The internalization of ligand by ICAM receptors may be a process of receptor recycling to control the receptor activity on the cell surface. Therefore, the receptor-ligand binding followed by endocytosis is one of the mechanisms for the termination of receptor activity (Almenar-Queralt et al., 1995).

This receptor-ligand internalization process is also found in other cell adhesion molecules such as integrin (Raub and Kuentzel, 1989) and VCAM-1 (Ricard et al., 1998).

Binding of FITC-cLAB.L to the D1 domain of ICAM-1 on both populations of PMA-activated Molt-3 T-cells was enhanced dramatically by the presence of a Ca^{2+} and Mg^{2+} ion mixture (Fig. 5c). However, Ca^{2+} or Mg^{2+} alone gave a less dramatic or no change in binding of FITC-cLAB.L to ICAMs, respectively. Ca^{2+} improved binding of FITC-cLAB.L to population-1 better than population-2. These results were supported by the suggestion that a section of I-domain binds to the ICAM-1 via divalent cations. Important residues on I-domain of LFA-1 for ICAM-1 binding have been proposed including Met¹⁴⁰, Glu¹⁴⁶, Thr²⁴³ and Ser²⁴⁵ (Huang and Springer, 1995), and recently Leu²⁰⁵ and Glu²⁴¹ (Edwards et al., 1998). Meanwhile, there are distinct but partially overlapping binding sites in the I-domain of LFA-1 for ICAM-1 and ICAM-3 (Binnerts et al., 1996) but none of the reported residues are part of the cLAB.L sequence. It was shown using CD, NMR and molecular modeling that cLAB.L can bind to Ca^{2+} and Mg^{2+} . The binding properties of cLAB.L were also evaluated using docking experiments between cLAB.L and the D1-domain of ICAM-1 in the presence and absence of divalent cations. The peptide can bind to ICAM-1 D1-domain via residue numbers 1 (Pen), 2 (Ile), 6 (Glu), and 10 (Ser).

Conclusion

In conclusion, direct binding between cLAB.L and ICAM-1 on the surface of Molt-3 T-cells is primarily via binding to ICAM-1, and to a lesser extent, via ICAM-3. The FITC-labeled peptide binding is expressed in bimodal distribution of cell population and it is saturable. The FITC-labeled peptide can be inhibited by the unlabeled cLAB.L, thereby indicating a receptor-mediated binding and no interference of FITC conjugation on the selectivity of peptide binding to the receptors. The peptide binding is influenced by a Ca^{2+} and Mg^{2+} mixture, and the peptide may also be internalized by ICAMs. Accordingly receptor-mediated internalization of cLAB.L, combined with other methods such as drug conjugation, may have useful applications in targeting leukocyte-related diseases.

EXAMPLE 2

This example confirmed that peptides derived from ICAM-1 could be conjugated with drugs and bind with cells expressing LFA-1 surface receptors. Furthermore, the conjugate was internalized and cells which internalized the conjugate

were killed in a concentration-dependent manner. Due to the peptide portion of the conjugate and its affinity for LFA-1 receptors, the drug portion of the conjugate was ineffective at killing cells which did not have LFA-1 receptors. Thus, peptide-drug conjugates were toxic toward selected targeted cells (i.e. cells expressing LFA-1 receptors) while the conjugate rendered the drug less toxic to non-targeted cells. Accordingly, similar conjugates will be effective in treating diseases related to LFA-1 expressing cells (T-cell-related disorders).

Materials and Methods:

Synthesis of MTX-cIBR conjugate:

Methotrexate (10 mg, 0.02 mmol, Sigma A6770) was dissolved in milliQ water and pH of the solution was adjusted to 7.0. EDC (0.02 mmol). Next, 10 mg (0.008 mmol) cIBR peptide (SEQ ID No. 8) (0.008 mmol) was added into the MTX solution and stored overnight at room temperature. The reaction was concentrated *in vacuo* and the crude product was purified with reversed-phase HPLC with C-18 column (12 μ M, 300 Å, 25 cm \times 21.5 mm i.d.) and UV detection at 220 nm. A flow rate of 10 ml/min was used using a gradient run using solvent A which consisted of 0.1% TFA in H₂O:acetonitrile (95:5) and solvent B which consisted of 100% acetonitrile. Fractions were collected and subjected to analytical HPLC in order to determine product purity. Fractions containing the desired product were then pooled, concentrated and lyophilized. The identity of the conjugate (MTX-cIBR) was confirmed by mass spectrometry ($M+1 = 1611.4$) and NMR.

Toxicity studies:

Toxicity studies were done by MTT assay, using both activated and non-activated MOLT-3 T-cells. Activation of the MOLT-3 T-cells was accomplished by using PMA (phorbol 12-myristate-13-acetate) at a final concentration of 0.2 μ M. Aliquots of 200 μ L of cell suspensions containing 2.92×10^5 cells/ml nonactivated, or 2.47×10^5 cells/ml activated MOLT-3 cells with 80% viability were plated onto a 48-well plate. Graded amounts of MTX, MTX-cIBR, and/or cIBR dissolved in PBS were then added to the plates and incubated for 48 hours at 37°C in a humidified 5% CO₂/95% air atmosphere. As a negative control, a treatment with 10mM iodoacetamide (IAA) for 48 hours was used to determine the minimal value of metabolic activity. A positive control of cells only was used to determine maximum metabolic activity. At the end of the incubation period, 40ul MTT (Sigma, St. Louis, MO) solution (5mg/ml PBS) was added to each well and further incubated for 2 hours at 37°C. Following incubation, cell suspensions were transferred to microcentrifuge tubes and centrifuged

for 3 minutes at 3,000 RPM before removing the supernatants. The formazan crystals produced were solubilized by the addition of 1ml of 0.04 N HCl in isopropanol and subsequently sonicated for 7-10 minutes. The microcentrifuge tubes were again spun for 3 minutes at 3,000 RPM and 100uL aliquots of supernatant placed onto a 96 well plate and the absorbance at 540-nm wavelength was immediately measured using a Titertek Multiskan MCC/340. Metabolic activity was calculated as follows, where O.D. is the measured optical density at 540nm.

$$\text{Metabolic activity} = - (\text{O.D.}_{\text{IAA}} - \text{O.D.}_{\text{sample}}) / (\text{O.D.}_{\text{MTT}} - \text{O.D.}_{\text{IAA}})$$

Toxicity studies using MDCK cells proceeded as follows. To a 48 well plate, 300uL aliquots of cell suspension (8×10^5 cell/ml) were grown to a confluent monolayer. Once a complete monolayer was reached, treatment with graded amounts of MTX, or MTX-cIBR were added and incubated as described. MTT assay was followed as above with one distinction: the cell monolayer, which remained attached to the plate after treatment, was scraped off and transferred to the microcentrifuge tube.

Results:

Results from toxicity studies show that the MTX-cIBR conjugate is toxic in a concentration dependent manner and is approximately 19 fold more toxic than MTX alone at a similar concentration. To demonstrate that this increase in toxicity was due to the cIBR binding to MOLT-3, it was shown that the toxicity of MTX-cIBR can be inhibited in a concentration dependent manner by adding increasing amounts of cIBR.

The specificity of this drug-peptide conjugate for cells expressing LFA-1 was demonstrated by comparing the toxicity in MDCK epithelial cells which do not express LFA-1. Treating MDCK cells with concentrations of MTX and MTX-cIBR which produced nearly completely toxic effects in T-cells showed that MTX-cIBR was approximately 5 times less toxic than MTX alone. Without LFA-1 receptors, the conjugate is not carried inside of the cells (e.g. it is not internalized). Therefore, this binding specificity provides a great deal of selectivity because only T-cells express LFA-1. Accordingly, T-cell-related diseases can be treated with such conjugates without the adverse side effects which generally accompany treatment with drugs effective against such diseases. Additionally, dosage levels may be lower due to the high potency of the drugs after internalization by the targeted cells.

Thus, the present invention can be used to conjugate drugs with peptides in order to treat leukocyte-related diseases such as asthma, inflammations, Chron's

Disease, rheumatoid arthritis, multiple sclerosis, ulcerative colitis, pemphigus vulgaris, pemphigoid, allergies, HIV-infections, and epidermolysis. Additionally, drugs effective in treating such diseases can be made less toxic to non-targeted cells as well as more potent against targeted cells by conjugating them with the peptides of the present invention. Another use of the present invention involves delivering HIV-protease inhibitors to T-cells. This method would help to avoid drug resistance against HIV-protease inhibitors, which has been rising in patients with AIDS. Because the replication of HIV is in T-cells, the use of peptides targeted to T-cells (i.e. peptides which bind to LFA-1 receptors) is appropriate for suppressing HIV replication. Additionally, the efflux pump receptors may be entirely avoided using the methods and peptide-drug conjugates of the present invention.

Procedures and teachings of the following references are hereby incorporated by reference:

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[illegible]

We claim:

1. A conjugate comprising a drug coupled with an isolated peptide sequence selected from the group consisting of peptide sequences derived from ICAM-1 and LFA-1.

2. The conjugate of claim 1, said isolated peptide sequence having from about 4-30 amino acid residues.

3. The conjugate of claim 1 said isolated peptide sequence selected from the group consisting of SEQ ID Nos. 1-8.

4. The conjugate of claim 3, said peptide differing from that of said isolated peptide sequence selected from the group consisting of SEQ ID. Nos. 1-8 due to a mutation event.

5. The conjugate of claim 4, said mutation event being selected from the group consisting of point mutations, deletions, insertions and rearrangements.

6. The conjugate of claim 1, said drug selected from a class of drugs consisting of antiinflammatory agents, antitumor agents, oligonucleotides, cytokines, enzyme inhibitors, and vasoregulator agents.

7. The conjugate of claim 1 said drug selected from the group consisting of methotrexate, lovastatin, taxol, ajmalicine, vinblastine, vincristine, cyclophosphamide, fluorouracil, idarubicin, ifosfamide, irinotecan, 6-mercaptopurine, mytomyins, mitoxantrone, paclitaxel, taxol, pentostatin, plicamycin, topotecan, fludarabine, etoposide, doxorubicin, doxotaxel, danorubicin, albuterol, and propidium.

8. The conjugate of claim 1, said drug being methotrexate.

9. The conjugate of claim 3, said isolated peptide sequence having at least about 50% homology with at least one of said SEQ ID Nos. 1-8.

10. A method of delivering drugs to leukocytes comprising the steps of:

forming a conjugate comprising a drug and an isolated peptide sequence selected from the group consisting of peptide sequences derived from ICAM-1 and LFA-1 sequences; contacting a leukocyte, epithelial cell, or endothelial cell with said conjugate; and causing said conjugate to be internalized within the leukocyte, epithelial cell, or endothelial cell.

11. The method of claim 10, said drug selected from a class of drugs consisting of antiinflammatory agents, antitumor agents, oligonucleotides, cytokines, enzyme inhibitors, and vasoregulator agents.

12. The method of claim 10, said drug being selected from the group consisting of methotrexate, lovastatin, taxol, ajmalicine, vinblastine, vincristine, cyclophosphamide, fluorouracil, idarubicin, ifosfamide, irinotecan, 6-mercaptopurine, mytomyocins, mitoxantrone, paclitaxel, taxol, pentostatin, plicamycin, topotecan, fludarabine, etoposide, doxorubicin, doxotaxel, danorubicin, albuterol, and propidium.

13. The method of claim 10, said drug being selected from the group consisting of methotrexate and doxorubicin.

14. The method of claim 10, said isolated peptide sequence having from about 4-30 amino acid residues.

15. The method of claim 10, said isolated peptide sequence being selected from the group consisting of SEQ ID Nos. 1-8.

16. The method of claim 15, said isolated peptide sequence having at least about 50% homology with at least one of said SEQ ID Nos. 1-8.

17. In a method of administering a drug to cells wherein the improvement comprises reducing the toxicity of the drug to non-targeted cells, said method comprising the step of coupling said drug with a peptide.

18. The method of claim 17, said drug selected from a class of drugs consisting of antiinflammatory agents, antitumor agents, oligonucleotides, cytokines, enzyme inhibitors, and vasoregulator agents.

5 19. The method of claim 17, said drug being selected from the group consisting of methotrexate, lovastatin, taxol, ajmalicine, vinblastine, vincristine, cyclophosphamide, fluorouracil, idarubicin, ifosfamide, irinotecan, 6-mercaptopurine, mytomyins, mitoxantrone, paclitaxel, taxol, pentostatin, plicamycin, topotecan, fludarabine, etoposide, doxorubicin, doxotaxel, danorubicin, albuterol, and propidium.

10 20. The method of claim 17, said drug being selected from the group consisting of methotrexate and doxorubicin.

15 21. The method of claim 17, said peptide having from about 4-30 amino acid residues.

22. The method of claim 17, said peptide being selected from the group consisting of SEQ ID Nos. 1-8.

20 23. The method of claim 21, said peptide having at least about 50% sequence homology with at least one of said SEQ ID Nos. 1-8.

24. A method of treating leukocyte-related diseases comprising the steps of:

25 conjugating a drug with a peptide sequence derived from the sequence of ICAM-1 in order to produce a peptide-drug conjugate, said peptide sequence being adapted to bind with LFA-1 receptors;
contacting said conjugate with a leukocyte;
causing said conjugate to be internalized by the leukocyte; and
30 causing said drug to kill the leukocyte.

25. The method of claim 24, said peptide sequence having from about 4-30 amino acid residues.

26. The method of claim 24, said peptide sequence being selected from the group consisting of sequences having at least about 50% sequence homology with at least one of SEQ ID Nos. 1-8.

5 27. The method of claim 24, said drug selected from a class of drugs consisting of antiinflammatory agents, antitumor agents, oligonucleotides, cytokines, enzyme inhibitors, and vasoregulator agents.

10 28. The method of claim 24, said drug selected from the group consisting of methotrexate, lovastatin, taxol, ajmalicine, vinblastine, vincristine, cyclophosphamide, fluorouracil, idarubicin, ifosfamide, irinotecan, 6-mercaptopurine, mytomyins, mitoxantrone, paclitaxel, taxol, pentostatin, plicamycin, topotecan, fludarabine, etoposide, doxorubicin, doxotaxel, danorubicin, albuterol, and propidium.

15 29. A method of treating an epithelial or endothelial cell-related disease comprising the steps of:

conjugating a drug with a peptide derived from LFA-1;
contacting said conjugate with a leukocyte, epithelial cell, or endothelial cell;
causing said conjugate to be internalized by the leukocyte, epithelial, or
20 endothelial cell; and
causing said conjugate to modulate the function of the contacted leukocyte, epithelial, or endothelial cell.

25 30. The method of claim 29, said disease being selected from the group consisting of asthma, inflammations, Chron's Disease, rheumatoid arthritis, multiple sclerosis, ulcerative colitis, pemphigus vulgaris, pephigoid, allergies, HIV-infections, and epidermolysis.

30 31. The method of claim 29, said disease being related to an increased expression of ICAM-1.

32. The method of claim 29, said peptide being adapted to bind with ICAM-1 receptors.

35 33. The method of claim 29, said peptide having from about 4-30 amino acid residues.

34. The method of claim 29, said peptide being selected from the group consisting of sequences having at least about 50% sequence homology with at least one of Sequence ID Nos. 1-8.

any other sequence having at least about 50% sequence homology with at least one of Sequence ID Nos. 1-8.

ABSTRACT

Peptides which bind to leukocytes are conjugated with drugs. Advantageously, the peptide-drug conjugates interfere with ICAM-1/LFA-1 interactions and are internalized by leukocytes, whereby the drug portion of the conjugate exerts potent toxic side effects against targeted cells with minimal adverse side effects. Preferred conjugates include 4-30 mer peptides derived from ICAM-1 or LFA-1 coupled with drugs effective against leukocyte-related diseases.

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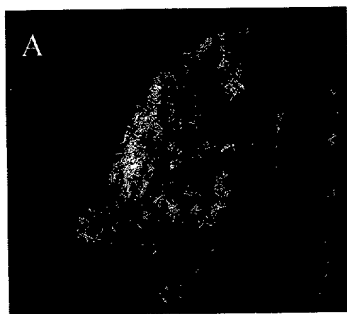


Fig. 6a

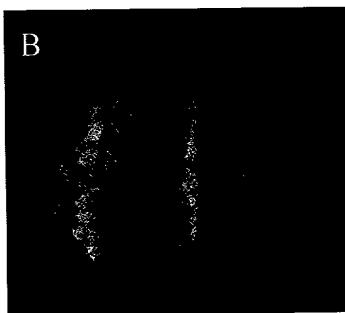


Fig. 6b

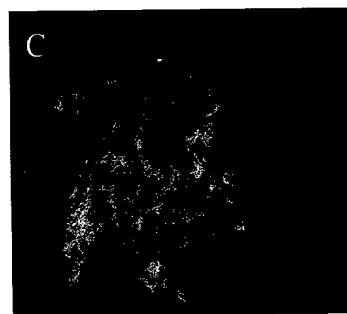


Fig. 6c

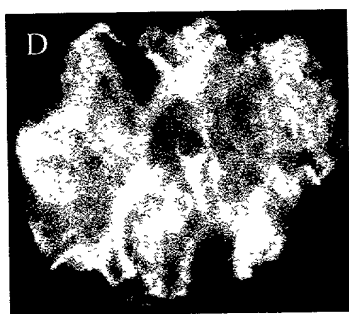


Fig. 6d



Fig. 6e

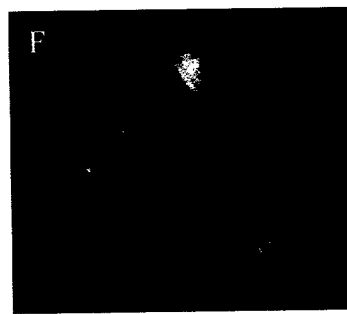


Fig. 6f



Fig. 6a

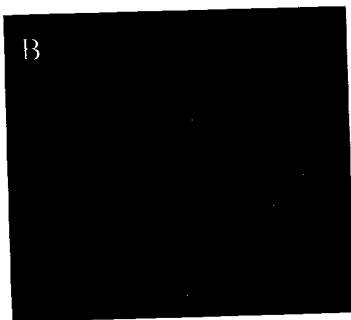


Fig. 6b

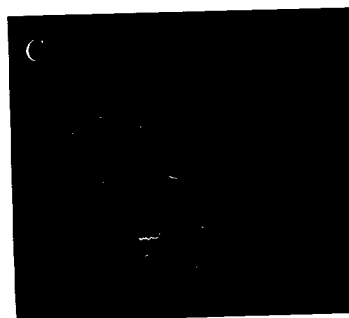


Fig. 6c

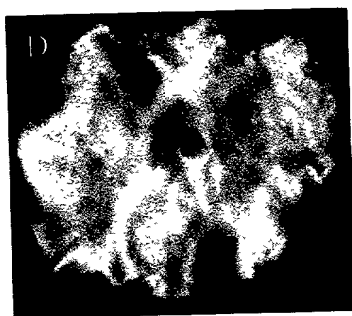


Fig. 6d



Fig. 6e

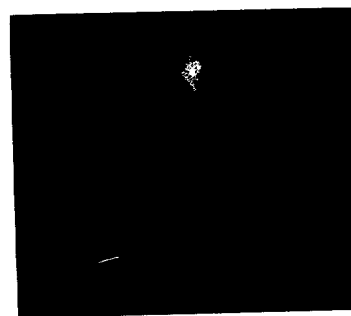


Fig. 6f

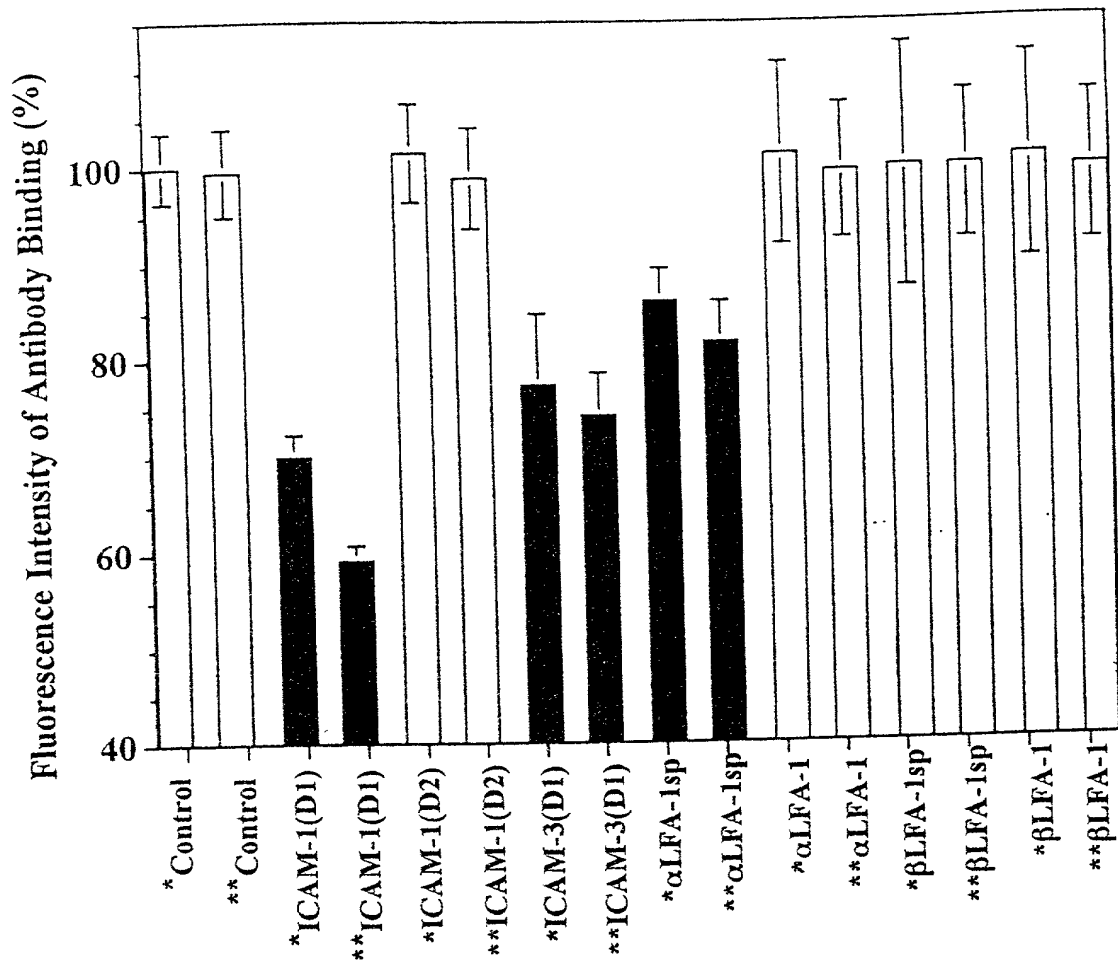


Fig. 1

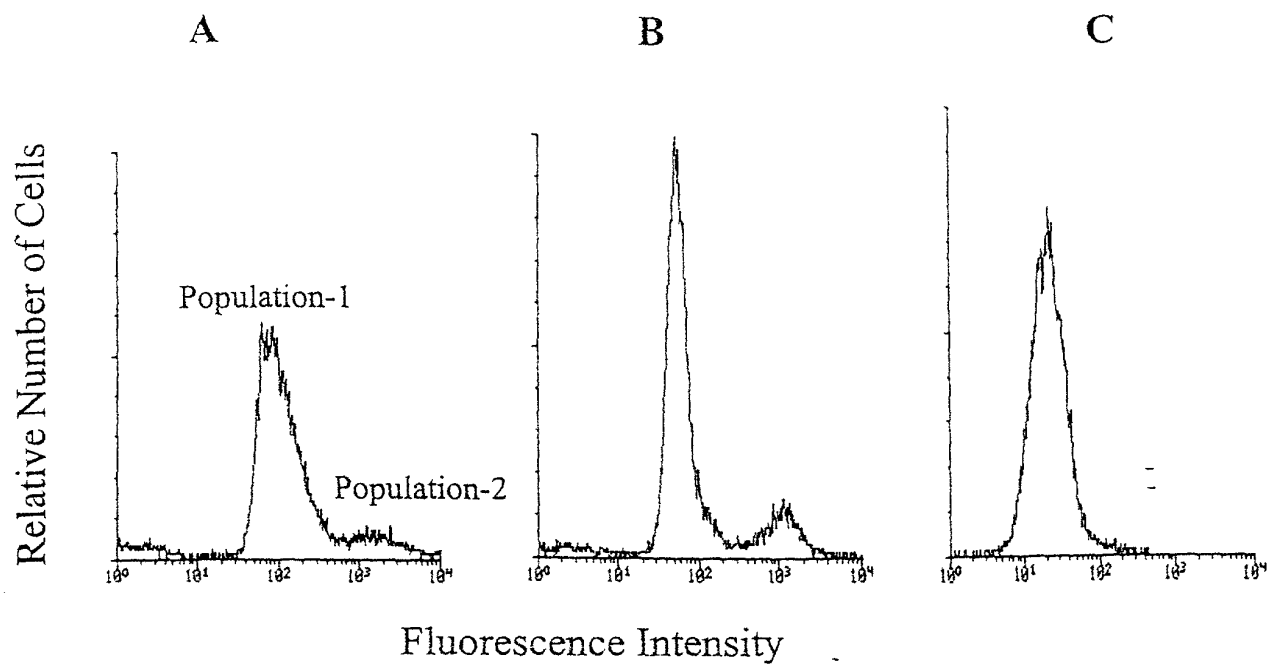


Fig. 2

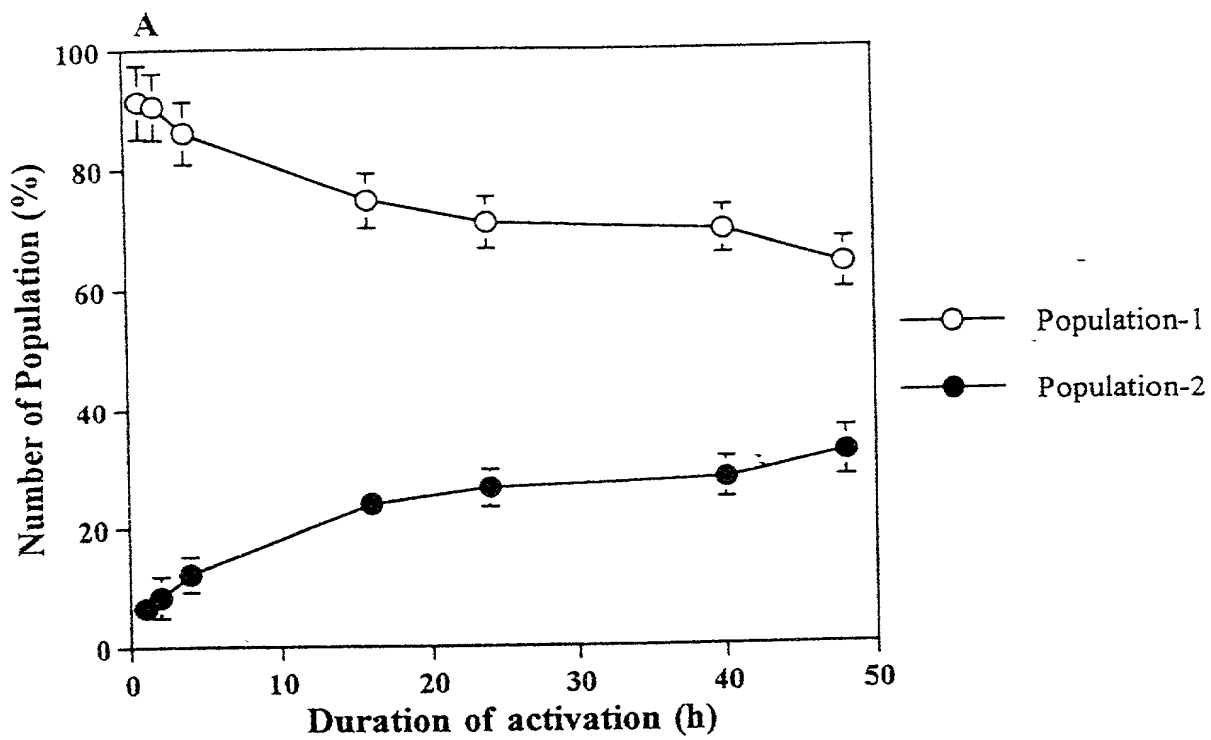


Fig. 4a

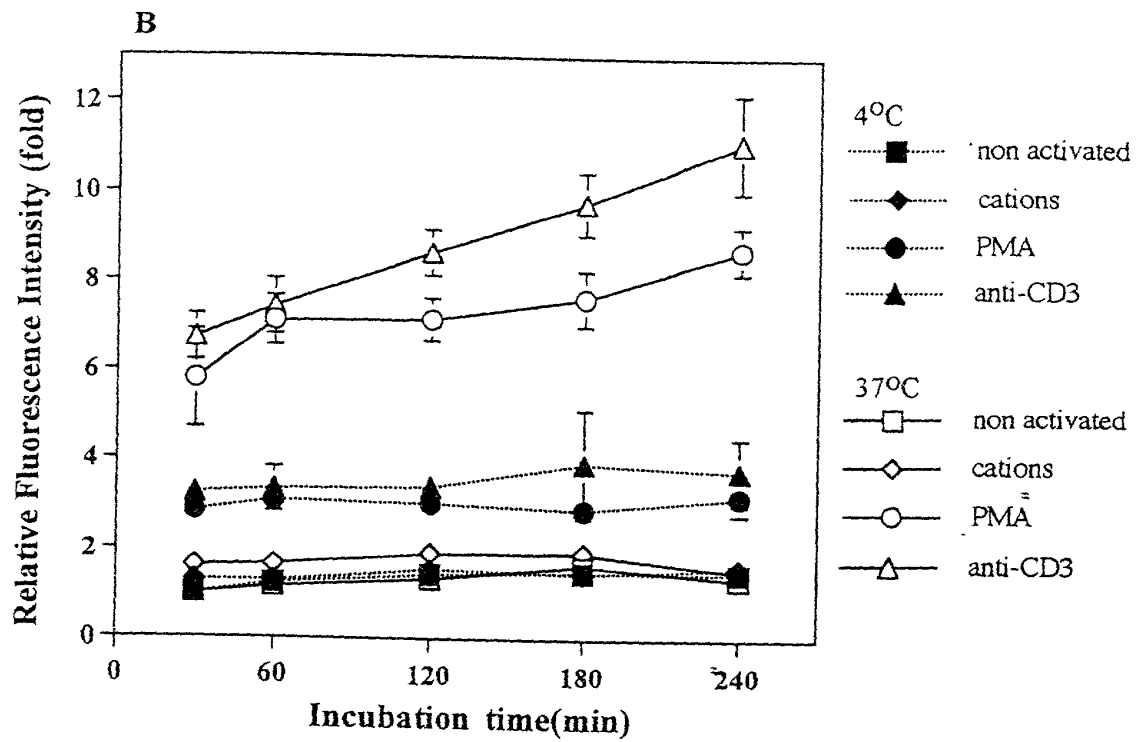


Fig. 4b

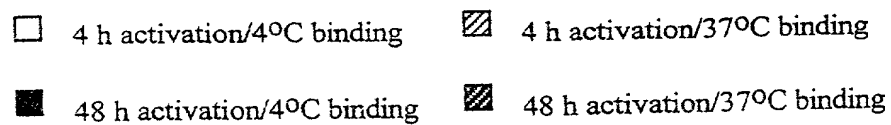
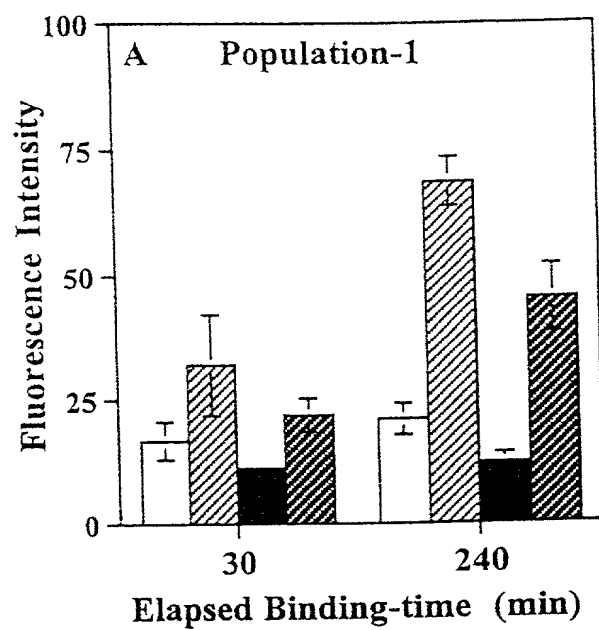
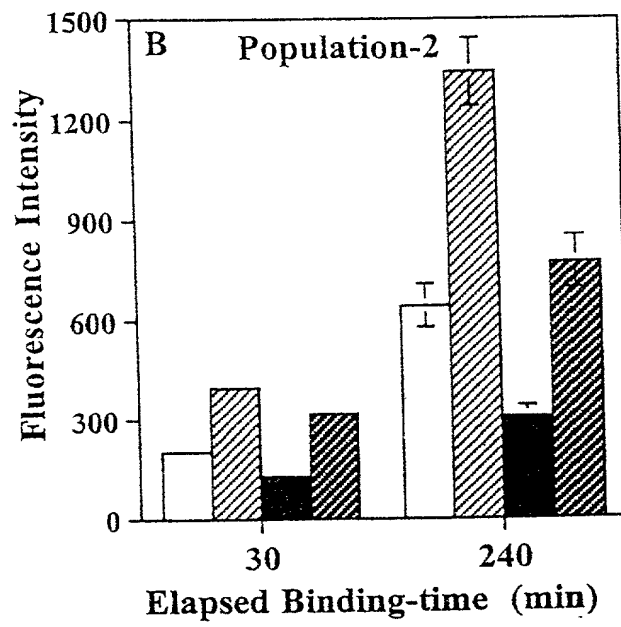


Fig. 5a



□ 4 h activation/4°C binding ▨ 4 h activation/37°C binding
 ■ 48 h activation/4°C binding ▩ 48 h activation/37°C binding

Fig. 5b

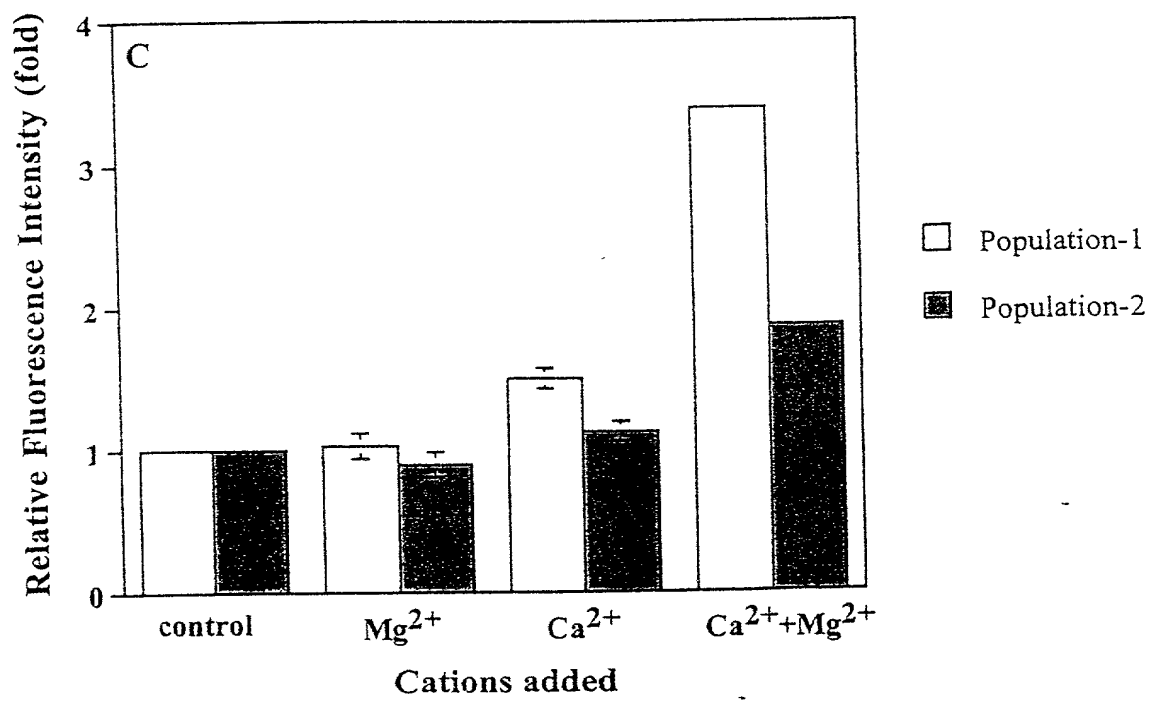


Fig. 5c

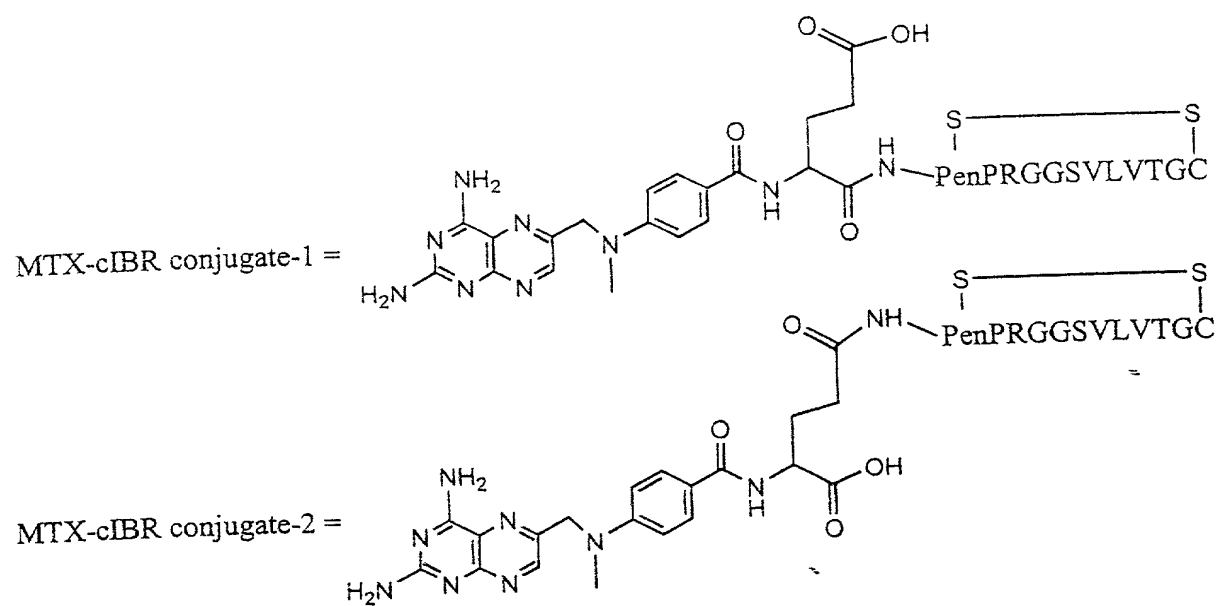


Fig. 7

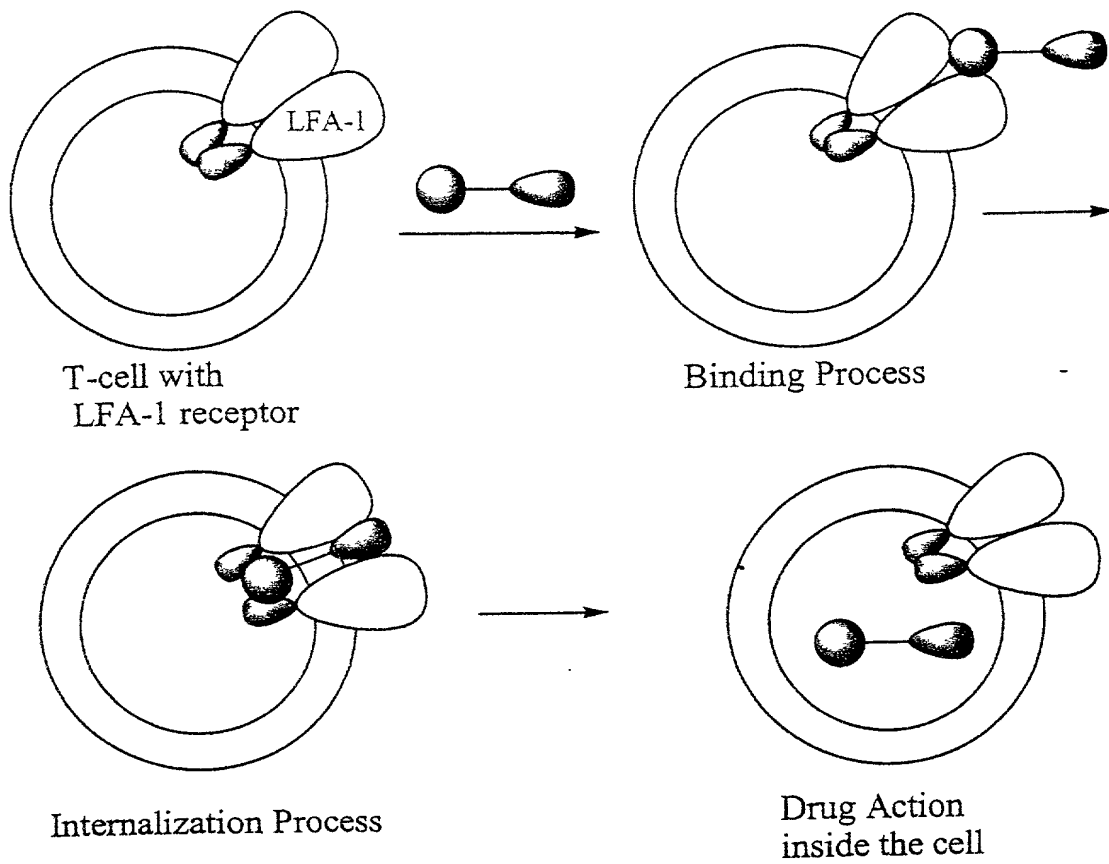
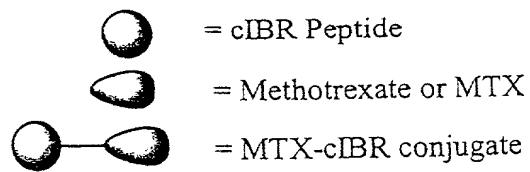


Fig. 8

Toxicity of MTX-clBR by MTT assay

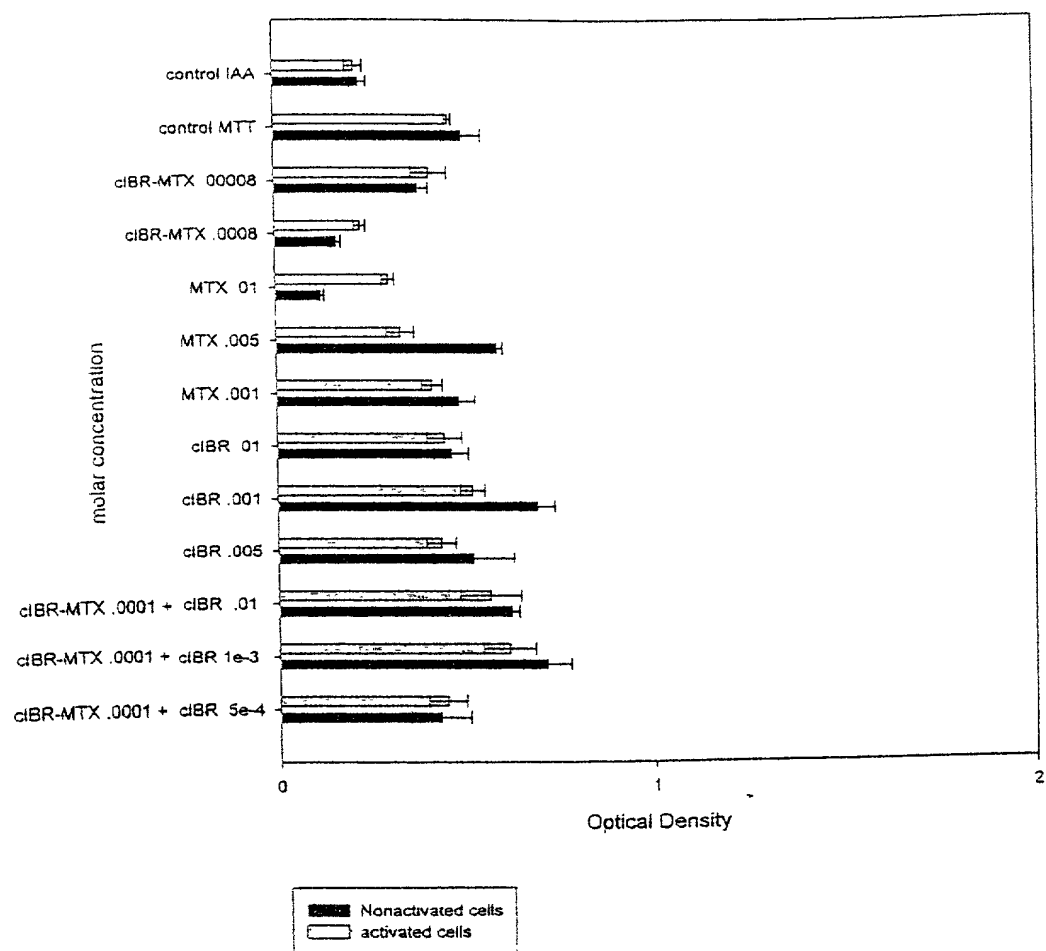


Fig. 9

metabolic activity after treatment

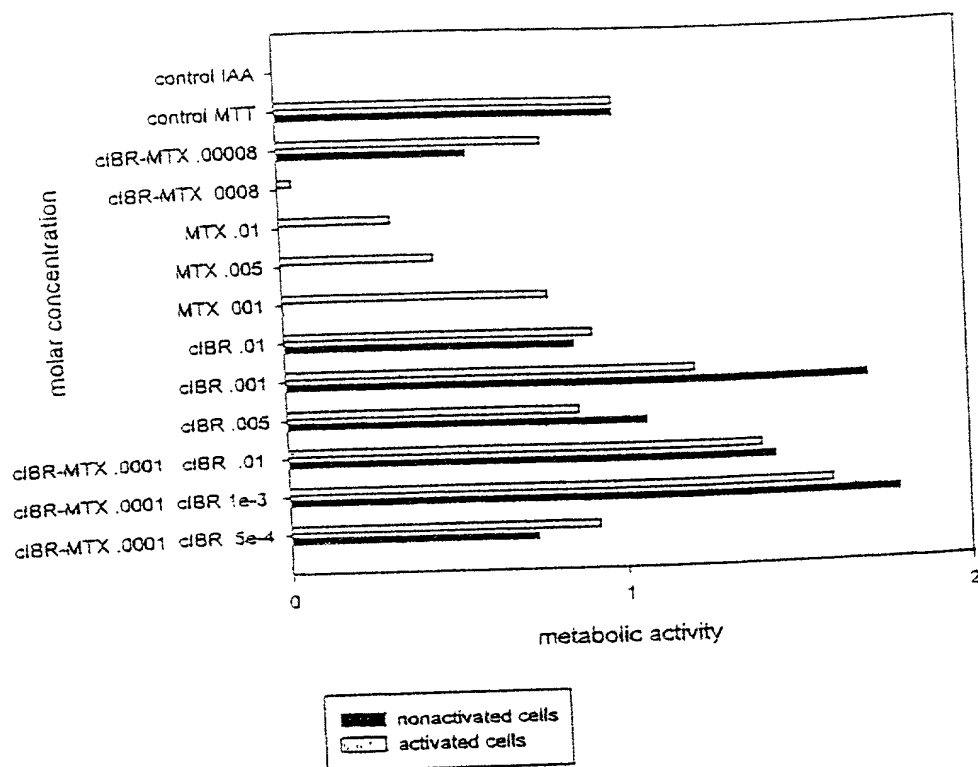


Fig. 10

As a below named inventor I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name, I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

CELL INTERNALIZED PEPTIDE-DRUG CONJUGATES

the specification of which: (complete (a), (b) or (c) for type of application)

REGULAR OR DESIGN APPLICATION

- (a) ☒ is attached hereto.
(b) ☐ was filed on as Application Serial No. _____ and was amended on _____ (if applicable).

PCT FILED APPLICATION ENTERING NATIONAL PHASE

- (c) ☐ was described and claimed in International Application No. _____ filed _____ and as amended on _____ (if any).

ACKNOWLEDGMENT OF REVIEW OF PAPERS AND DUTY OF CANDOR

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56(a).

- ☐ In compliance with this duty there is attached an information disclosure statement. 37 CFR 1.97.

PRIORITY CLAIM

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:
(complete (d) or (e))

- (d) ☒ no such applications have been filed.
(e) ☐ such applications have been filed as follows

EARLIEST FOREIGN APPLICATION(S), IF ANY FILED WITHIN 12 MONTHS PRIOR TO SAID APPLICATION

Country	Application No.	Date of Filing	Date of Issue	Priority Claimed
				<input type="checkbox"/> YES <input type="checkbox"/> NO
				<input type="checkbox"/> YES <input type="checkbox"/> NO
				<input type="checkbox"/> YES <input type="checkbox"/> NO

ALL FOREIGN APPLICATION(S), IF ANY FILED MORE THAN 12 MONTHS PRIOR TO SAID APPLICATION

PROVISIONAL

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States application(s) listed below:

Application Serial No.	Filing Date	Status (patented, pending, abandoned)
------------------------	-------------	---------------------------------------

CONTINUATION-IN-PART

(Complete This Part Only If This Is A Continuation-In-Part Application)

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56(a), which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application:

Application Serial No.	Filing Date	Status (patented, pending, abandoned)
------------------------	-------------	---------------------------------------

POWER OF ATTORNEY



As a named inventor, I hereby appoint the following attorney(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

Robert D. Hovey	19,223	Thomas B. Luebbering	37,874
Warren N. Williams	19,156	Andrew G. Colombo	40,565
Stephen D. Timmons	26,513	Tracy L. Bornman	42,347
John M. Collins	26,262	Tracey S. Truitt	43,205
Thomas H. Van Hoozer	32,761		

SEND CORRESPONDENCE TO:
HOVEY, WILLIAMS, TIMMONS & COLLINS
2405 Grand, Suite 400
Kansas City, Missouri 64108

DIRECT TELEPHONE CALLS TO:
(816) 474-9050

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor		Teruna J. Siahaan	
Inventor's Signature 			
Date	07/18/00	Country of Citizenship	USA
Residence 2912 Iris Lane, Lawrence, Kansas 66047			
Post Office Address 2912 Iris Lane, Lawrence, Kansas 66047			
Full name of second joint inventor, if any		Helena Yusuf-Makagiansar	
Inventor's Signature 			
Date	07/18/2000	Country of Citizenship	Indonesia
Residence E206 Cornish Square, Lawrence, Kansas 66049			
Post Office Address E206 Cornish Square, Lawrence, Kansas 66049			

Full name of third joint inventor, if any		Meagan Anderson	
Inventor's Signature <i>Meagan Anderson</i>			
Date <i>7-18-2000</i>		Country of Citizenship USA	
Residence		740 N. Clinton, Olathe, Kansas	
Post Office Address		740 N. Clinton, Olathe, Kansas	
Full name of fourth inventor, if any		Rong "Christine" Xu	
Inventor's Signature <i>Rong Xu</i>			
Date <i>07/18/2000</i>		Country of Citizenship Singapore <i>CRX</i>	
Residence		2350 Ridge Court, Apt. 21, Lawrence, Kansas 66046	
Post Office Address		2350 Ridge Court, Apt. 21, Lawrence, Kansas 66046	

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<110> SIAHAAN, TERUNA J
YUSUF-MAKAGIANSAR, HELENA
ANDERSON, MEAGAN
XU, RONG

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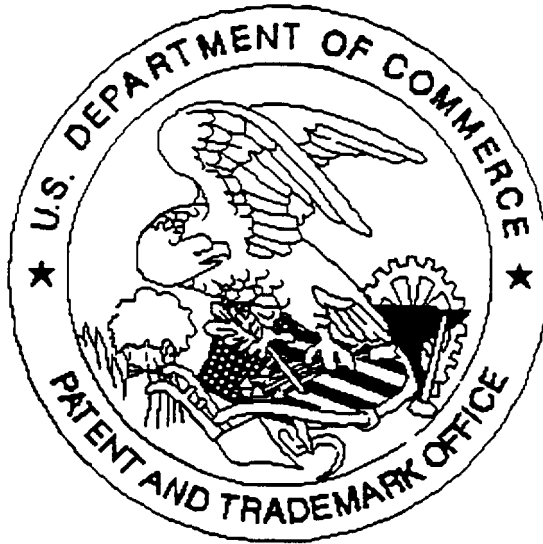
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